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(54) Title: ENZYMATIC TREATMENT OF HIDES AND LEATHER

(57) Abstract: Treatment of leather or animal skins with a transglutaminase (TG), preferably together with a glutamine and/or lysine containing polymer or oligomer, such as keratin or casein, has a beneficial effect on the subsequent dyeing and colour properties of leather. The uptake of protein or enzyme is improved when the hide or leather is dried prior to treatment with a solution of protein or enzyme. Transglutaminase treatment is also shown to improve resistance to abrasion and collagenase.

ENZYMATIC TREATMENT OF HIDES AND LEATHER

Background of the Invention

5 This invention is concerned with procedures for treating hides and leather with enzymes. In particular, the invention concerns a procedure to improve the take-up of enzymes in leather preparative processes in which enzymes and proteins are used, and also the use of specific enzymes and proteins to improve the dyeing of hides and leather.

10 The traditional method of producing leather from animal hides has remained relatively unchanged for centuries. The tanning process is used to increase the hydrothermal stability of the collagen via cross-linking effects and generally uses chromium salts or vegetable extracts. Hides are prepared for tanning by soaking, 15 liming, deliming, degreasing and bating.

The use of enzymes in the leather making process has become widespread, with a variety of enzymes used in the soaking, liming and degreasing stages. One of the main problems with enzymatic treatments is ensuring thorough and even penetration of the 20 enzyme throughout the entire hide. One object of the present invention is to overcome that problem by providing a method of treatment that allows even and efficient uptake of enzymes.

25 Although some colour may be imparted to leather by the tanning agents during tanning, leather is usually dyed to improve its appearance and to make it saleable. A variety of dyeing procedures are in commercial use, the most commonly used being drum and spray application. The majority of leather dyes are anionic, of the acid and direct types. Fixation of these conventional leather dyes is favoured under acid conditions due to protonation of amino groups in the collagen matrix. The process of 30 fixation is reversible and raising the pH of the leather causes stripping of the dyestuff. The dyeing mechanism involves salt linkages, hydrogen bonds, Van der Waal's bonds and other electrostatic attractions which are weak and easy to break. The weakness of these electrostatic attractions and the reversibility of the dyeing mechanism coupled with the very high dye offers used means that dyed leathers generally have very poor 35 colour fastness properties, particularly when compared to some man-made fibres.

Another technique uses a cationic fixative as an after treatment to the dyeing process. These cationic fixatives form an insoluble complex with the anionic dyestuff in the leather structure which prevents dissolution of loose dyestuff. Use of these fixatives can, thus, significantly reduce dye migration. However, although these complexes 5 have low aqueous solubility they are not chemically bound to the leather substrate and are frequently easily removed by rubbing, such as might occur during wear, resulting in transfer of colour to adjacent items of clothing, or during domestic laundering processes. The complexes formed between the anionic dyestuffs and the cationic fixatives are also easily removed with organic solvents thus reducing the dry- 10 cleanability of the leather. Poor colour fastness of leather is a major quality issue for the leather industry being one of the most common sources of customer complaint.

One method which has been used to effect improvements in colour fastness of dyed leathers is through the use of reactive dyestuffs. These dyestuffs are essentially 15 similar to conventional leather dyestuffs, being anionic. However, the one important difference is that they contain one or more groupings, integral with the dye molecule, which are capable of forming covalent bonds between the dye and the substrate. Use of these materials has shown potential for producing dyed leathers with very high performance levels in terms of colour fastness. However, certain disadvantages are 20 associated with the use of these dyes. Leather has a limited number of groups available in the collagen matrix with which the reactive dye can react. Also, during the reactive dyeing process, as well as the desired reaction with the substrate, a competing reaction also occurs with the solvent, in this case water. This means that some of the dyestuff is lost during the process, due to hydrolysis of the reactive 25 groups, and is washed out into the effluent. As well as the obvious economic implications of losing part of the dye offer there are also serious environmental implications associated with this class of dye due to the hydrolysed dye polluting the effluent. Due to the cationic nature of the leather substrate, complete washing off of this hydrolysed dyestuff is also extremely difficult. If this washing off process is not 30 carried out effectively and some hydrolysed dye is allowed to remain in the leather, then this can give rise to poor wet fastness and the benefit of the covalently bonded dye is lost. These limitations have meant that reactive dyes have not found widespread usage within the industry.

35 Due to the high level of customer complaints arising as a result of poor colour fastness of dyed leathers there remains a need for viable process capable of

significantly improving fastness properties. One object of the present invention is to overcome that problem by providing a method of dyeing that use enzymes as a dyeing aid.

5 **Summary of the Invention**

One aspect of this invention is based on the finding that treatment of leather or animal skins with a transglutaminase (TG) and glutamine and/or lysine containing polymers or oligomers has a beneficial effect on the subsequent dyeing and colour properties of 10 leather.

Accordingly the present invention provides a process for the treatment of leather, especially in preparation for dyeing, in which leather or animal skins are treated with a transglutaminase (TG) and optionally a glutamine and/or lysine containing polymer 15 or oligomer.

Typically the treatment is carried out by conventional leather processing techniques, for example drumming the leather or skins in an aqueous bath containing an effective amount of the TG enzyme and glutamine and/or lysine containing polymer or 20 oligomer. After this treatment, dyeing may be carried out conventionally, for example by adding the dyestuff to the bath in the drum.

Another aspect of the present invention is based on the finding that, in treatments requiring that proteins or enzymes are absorbed into hides or leather, the uptake of 25 protein or enzyme is improved when the hide or leather is dried prior to treatment with a solution of protein or enzyme.

Accordingly the present invention provides a process for the treatment of animal hides or leather in which the hide or leather is treated with a solution of protein or enzyme 30 so that the protein or enzyme is absorbed into the hide or leather characterised in that the hide or leather is dried prior to treatment with the protein or enzyme, and the solution of protein or enzyme is applied to the dried hide or leather.

Drying techniques for use in the process of this invention include air drying, freeze 35 drying, oven drying, vacuum oven drying, and solvent dehydration.

Typically the enzyme or protein treatment is carried out by conventional leather processing techniques, for example soaking or drumming the dried hides or leather in an aqueous bath containing an effective amount of the enzyme or protein.

5 The drying treatment is advantageously used to enhance the uptake of transglutaminase (TG) and a glutamine and/or lysine containing polymer or oligomer in the dyeing process of the invention.

Brief Description of the Drawings

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Figure 1 is a photomicrograph showing fluorescent confocal microscopy detection of biotinylated guinea pig liver transglutaminase (GPL tTG) (Cy5 streptavidin detection) after incubation with bated bovine hide. **A** is a control without biotinylated GPL tTG, **B** is wet bated bovine hide, **C** is freeze dried bated bovine hide. The total thickness of 15 hide was 5mm.

Figure 2 is a photomicrograph by confocal microscopy showing the pattern of GPL tTG mediated incorporation of biotin cadaverine (Cy5 streptavidin detection) into bated bovine hide. **A** is a control without GPL tTG, **B** is incorporation after soaking 20 into wet hide and **C** shows incorporation after soaking into freeze dried hide.

Figure 3 is a photomicrograph showing fluorescent confocal microscopy detection of biotinylated microbial transglutaminase (mTG) (Cy5 streptavidin detection) after incubation with bated bovine hide. **A** is a control without biotinylated mTG, **B** is wet 25 bated bovine hide, **C** is freeze dried bated bovine hide. The total thickness of hide was 5mm.

Figure 4 is a photomicrograph by confocal microscopy showing the pattern of mTG mediated incorporation of biotin cadaverine (Cy5 streptavidin detection) into bated 30 hide. **A** is a control without mTG, **B** is incorporation after soaking into wet hide and **C** shows incorporation after soaking into freeze dried hide.

Figure 5 is a graphical qualitative assessment of penetration of trypsin, mTG and GPL tTG into wet and freeze dried bated bovine hide. Measurements were made from 35 confocal microscopy images at 15min and 60min time points through both the grain and flesh sides.

Figure 6 is a graphical qualitative assessment of penetration of trypsin, mTG and GPL tTG into wet and freeze dried bovine rawhide. Measurements were made from confocal microscopy images at a 60min time point through both the grain and flesh sides.

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Figure 7 is a graphical qualitative assessment of penetration of trypsin, mTG and GPL tTG into wet and freeze dried delimed bovine hide. Measurements were made from confocal microscopy images at a 60min time point through both the grain and flesh sides.

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Figure 8 is a photomicrograph showing biotinylated transglutaminase (Cy5 streptavidin detection) penetration into crust leather after 60min. **A:** GPL tTG, **B:** mTG

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Figure 9 is a photomicrograph by confocal microscopy showing the pattern of mTG mediated incorporation of biotin cadaverine (Cy5 streptavidin detection) into crust leather. **A:** No biotinylated mTG control. **B:** biotinylated mTG soaked into crust leather for 60min.

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Figure 10 is a photomicrograph showing distribution of crosslink transversely across a section of mTG treated bated bovine hide. 1mm sections were cut from each region shown above for analysis. The image shows the corresponding pattern of biotin cadaverine incorporation by mTG as determined previously.

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Figure 11 is a photomicrograph showing biotinylated GPL tTG (Cy5 streptavidin detection) penetration into pickled goat skins after 60min. **A:** GPL tTG penetration into wet pickled goat skin, **B:** GPL tTG penetration into freeze dried pickled goat skin.

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Figure 12 is a photomicrograph showing biotinylated mTG (Cy5 streptavidin detection) penetration into pickled goat skins after 60min. **A:** mTG penetration into wet pickled goat skin, **B:** mTG penetration into freeze dried pickled goat skin.

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Figure 13 is a photomicrograph showing biotinylated GPL tTG (Cy5 streptavidin detection) penetration into pickled wool-on slink skins after 60min. **A:** GPL tTG penetration into wet pickled wool-on slink skin, **B:** GPL tTG penetration into freeze

dried pickled wool-on slink skin

5 **Figure 14** shows biotinylated mTG (Cy5 streptavidin detection) penetration into pickled wool-on slink skins after 60min. A: mTG penetration into wet pickled wool-on slink skin, B: mTG penetration into freeze dried pickled wool-on slink skin.

10 **Figure 15** shows distribution of biotinylated mTG (Cy5 streptavidin detection) after uptake into acetone dried bated bovine hide.

15 **Figure 16** is a graph showing dye release from freeze-dried bated leather samples treated with TG and/or biopolymer (putrescine and/or keratin) by perspiration solution treatment vs lightness of the leather grain surface (n=4).

20 **Figure 17** is a graph showing dye release from bated leather samples treated with TG and/or biopolymer (putrescine and/or keratin) by perspiration solution treatment vs lightness of the leather flesh surface (n=4).

25 **Figure 18** is a graph showing dye release from crust leather samples treated with TG and/or biopolymer (putrescine and/or keratin) by perspiration solution treatment vs lightness of the leather grain surface (n=4).

30 **Figure 19** is a graph showing dye release from bated leather samples treated with TG and/or biopolymer (putrescine and/or keratin) by perspiration solution treatment vs lightness of the leather flesh surface (n=4).

35 **Figure 20** is a graph showing depth of shade before and after treatment with an artificial perspiration solution, of the grain surface of leathers dyed with an acid dye after treatment with TG + keratin, keratin or TG. Values relative to an untreated, dyed control before and after treatment with artificial perspiration solution.

40 **Figure 21** is a graph showing depth of shade before and after treatment with a wash fastness solution, of the grain surface of leathers dyed with an acid dye after treatment with TG + keratin, keratin or TG. Values relative to an untreated, dyed control before and after treatment with wash fastness solution.

Figure 22 is a graph showing depth of shade before and after treatment with an artificial perspiration solution, of the grain surface of leathers dyed with a reactive dye after treatment with TG + keratin, keratin or TG. Values relative to an untreated, dyed control before and after treatment with artificial perspiration solution.

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Figure 23 is a graph showing depth of shade before and after treatment with a wash fastness solution, of the grain surface of leathers dyed with a reactive dye after treatment with TG + keratin, keratin or TG. Values relative to an untreated, dyed control before and after treatment with wash fastness solution.

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Figure 24 is a bar chart showing mTG/keratin effect on wool resistance to abrasion after 1000 rubbing cycles (n=8) - higher percentage of wool loss indicates less resistance to abrasion.

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Figure 25 is a bar chart showing mTG mediated crosslinking effect on resistance towards microbial collagenase - higher values of hydroxyproline indicate less resistance towards microbial collagenase.

Detailed Description of the Invention

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One aspect of the present invention originates in experiments to determine whether enzymes could penetrate into bated bovine hide. Biotinylated trypsin, microbial transglutaminase (mTG) and guinea pig liver transglutaminase (GPL tTG) were used to track their localisation after incubation for 2 hours. Surprisingly, preliminary data showed that biotinylated GPL tTG did not penetrate the hide at all and biotinylated trypsin and mTG penetrated to a depth of less than 1mm.

25

A search for a method of facilitated uptake of enzymes into bated bovine hide resulted in the finding that freeze drying of the bated hide followed by rehydration with enzyme-containing solutions showed a dramatic improvement in the uptake of trypsin and mTG with full penetration through the section within 15min under the test conditions. However, GPL tTG did not penetrate to any significant degree under the same conditions. This suggests that the ability of the protein to penetrate shows a dependence on size.

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Improvements in uptake by freeze drying were not as great with bovine rawhide or delimed hide, suggesting that the structure of the hide is more open after bating or that there are components of the hide that are removed during bating that allow larger proteins to enter.

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To determine the ability of transglutaminases to crosslink bated bovine hide after enhanced uptake by using freeze drying, a range of concentrations of mTG and GPL tTG were used. It was found that mTG was very effective at crosslinking freeze dried bated bovine hide, whilst GPL tTG was less effective because of reduced penetration.

10 The amount of crosslink incorporated by $100\mu\text{gml}^{-1}$ mTG into intact hide in 2 hours was actually greater than that incorporated by 1mgml^{-1} GPL tTG into bated bovine hide powder which had been traditionally limed and delimed. This disparity may be explained by the presence of inhibitory amounts of NH_4^+ in the bated hide used previously. However GPL tTG may still be suitable under some conditiond, for
15 eample for the selective modification of surface substrates.

Analysis of crosslink distribution showed that there was fairly even incorporation of mTG throughout the whole bated hide section. This indicated that sufficient mTG had reached the centre of the hide to incorporate the maximum possible amount of
20 crosslink. The pattern of biotin cadaverine incorporation under the same conditions showed more intense staining at each surface than towards the middle.

Another stage in the leather making process that is a candidate for transglutaminase treatment is crust leather (air dried leather). Based on the effect of freeze-drying, the
25 air dried leather has potential to be an ideal substrate for enhanced uptake although by this stage, pickling, chrome tanning and fat-liquoring have taken place.

Based on these findings, the present invention was developed. This invention provides a process for the treatment of animal hides or leather in which the hide or
30 leather is dried prior to treatment with a solution of protein or enzyme, and a solution of protein or enzyme is applied to the dried hide or leather so that the protein or enzyme is absorbed into the hide or leather.

The references herein to hides is made in its broadest sense and includes for example
35 raw hides and skins that have received one or more treatments towards the production

of leather, for example liming, deliming, bating, degreasing, pickling, etc. Similarly leather is used broadly to mean not only finished leather but partly processed leather.

Drying techniques for use in the process of this invention include air drying, freeze 5 drying, oven drying, vacuum oven drying, and solvent dehydration. Acetone drying has been shown to be particularly effective, although other solvents could be used, for example methylethylketone, acetonitrile, lower alcohols and diethyl ether. Freeze drying has been found to be especially effective. Commercial freeze-drying apparatus may be used in accordance with the manufacturer's parameters, but any combination 10 of lowered temperature and pressure may be used that maintains the frozen state of the hide or leather and allows sublimation of water to occur. Oven drying may typically be carried out at temperatures between normal ambient temperature and the shrinkage temperature of the hide or leather to be processed and vacuum drying with a similar range of temperatures and pressures below the ambient atmospheric pressure. 15 Hides and leathers may also be simply air dried under ambient conditions of temperature and pressure.

Enzymes that may find use in leather processing in accordance with this invention include transferases, hydrolases, lysases, and isomerases. Examples of such enzymes 20 are: protein-glutamine gamma-glutamyltransferases (transglutaminases EC 2.3.2.13) for improving the dyeing characteristics of leather; proteases such as trypsin (EC 3.1.21.4) or elastase (EC 3.4.21.36) for removal of non-collagenous proteins; or hyaluronidase (EC 3.2.1.35) for removal of hyaluronic acid and dermatans; chondroitinase (EC 4.2.2.4) for the removal of chondroitin and dermatan sulphates; 25 protein disulphide isomerases (EC 5.3.4.1) for the reorganisation or derivatisation of disulphide bonds in leather.

Proteins that may be used in leather processing include casein, which may be used for the finishing of leathers (current application) and keratin or silk, which may be used 30 to improve the dyeing characteristics of leathers as well as the finish, with or without addition of transglutaminase.

Typically the enzyme or protein treatment is carried out by conventional leather 35 processing techniques, for example soaking or drumming the dried hides or leather in an aqueous bath containing an effective amount of the enzyme or protein.

The other aspect of the present invention is based on experiments to improve the quality of dyed leather by increasing the number of available binding sites for subsequently applied dye. It was found that treating skins or leather with a transglutaminase (TG), typically as a microbial transglutaminase (mTG), and

5 preferably together with a glutamine and/or lysine containing polymer or oligomer, results in the incorporation of a polymer or oligomer containing peptide bound glutamine and/or lysine into the leather and thereby increases the number of available binding sites for the dye.

10 The pre-enzyme treatment drying process of the first aspect of this invention, may be used to prepare hides or leather for dyeing in accordance with the second aspect of this invention, by treating dried skins or leather with the transglutaminase (TG) and glutamine and/or lysine containing polymer or oligomer.

15 It has been found that drying of animal hides at the bating stage of leather processing significantly enhances the uptake of proteins such as enzymes and allows almost full thickness penetration within a relatively short time period. The drying methods of air drying, freeze drying and solvent drying have been investigated and all found to be suitable for this purpose. However it should be noted that the dyeing process may

20 also be used by applying a transglutaminase (TG), preferably together with a glutamine and/or lysine containing polymer or oligomer, to wet hides or leather without a preliminary drying step, or applied to crust leather, and still obtain an improvement over the conventional dyeing which does not use TG.

25 The polymer or oligomer, which is preferably rich in either lysine or glutamine or both, may be a protein such as keratin or casein. Such biopolymers are commercially available in powdered form which allows them to be easily dissolved in an aqueous bath.

30 Transglutaminase used in the present invention preferably has a smaller molecular size than guinea pig liver transglutaminase, for example less than about 80 kiloDaltons, to improve penetration times and avoid lengthy treatments. Microbial transglutaminase (eg at about 38 kDa) has been found to be especially effective for use in this invention, and may be obtained by fermentation of a suitable

35 microorganism, such as *Streptovorticillium mobaraense*. However the source is not critical and transglutaminase obtained from plants, animals and fungi may be used,

bearing in mind that a smaller molecular size will improve penetration and reduce treatment times. The enzyme is currently available commercially in powdered form, e.g. Activa WM from Ajinomoto Inc. Typically in the commercial form the enzyme is dispersed in an inert carrier, such as a dextrin, e.g. a maltodextrin. The carrier may 5 be removed before use in this invention, if desired.

It is also advantageous to use a polyamine, for example diamines with two N-terminal amines with an aliphatic carbon chain of at least four units [$\text{H}_2\text{N}-(\text{CH}_2)_n-\text{NH}_2$ with $n \geq 4$], as an optional additional agent in the treatment process of this invention. The 10 polyamine is believed to form transglutaminase mediated amide linkages with the peptide bound glutamine and so is also incorporated into the leather to provide additional dye-binding sites. These glutamine residues can be in the biopolymer or in the collagen of the leather itself. The addition of bifunctional amines can also be used to facilitate crosslinking of collagen with either itself or other peptides or proteins and 15 also to alter the charge properties of leather. For example, suitable compounds include diamines such as putrescine, cadaverine, 1,6-diamine-hexane, 1,7-diamine-heptane, and polyamines such as spermine and spermidine.

The enzyme treatment of the invention and the dyeing operation may be carried out in 20 accordance with conventional processing. For example pre-neutralised/retanned leather is loaded into an aqueous bath containing the transglutaminase and biopolymer and optionally the polyamine, and drummed to achieve adequate penetration. In some instances, it may be advantageous to perform the fat-liquoring step after the transglutaminase treatment in case the fat-liquoring components slow the penetration 25 of the enzyme. Alternatively suitably increased drumming times can be used to obtain effective penetration. Then, optionally after a washing/rinsing step, the dyestuff is added and similarly drummed. The process can be carried out within a range of pH 5 - 9 and temperature of 4 - 50 °C.

30 The invention is applicable to direct, acid and reactive dyes, which are applied conventionally in accordance with usual dyeing protocols. For example an acid dye may be added to the aqueous bath after the pH is adjusted to around 8.5, and the dyeing is completed by reducing the pH to about 3.5.

35 In addition to the above described improvement for dye quality, it has been found that the TG or TG/biopolymer treatment advantageously improves other properties of the treated hides or leather. In particular there are improvements in abrasion resistance,

especially for wool-on skins, and in resistance to collagenase. The improved abrasion resistance is of especial importance for wool-on skins since it significantly reduces the loss of wool during abrasion. resistance to collagenase improves the resistance of treated skins to microbial attack. These further advantages are obtainable whether or 5 not the hides or leather are dyed after the TG or TG/biopolymer treatment, and whether or not a preliminary drying step is used.

Accordingly, the use of TG or TG/biopolymer to improve abrasion resistance and to improve collagenase resistance form further aspects of the invention, independently of 10 the use of TG or TG/biopolymer to improve dyeing quality.

The implementation of the invention is illustrated, by way of example only, in the following Examples.

15 **Methods and Materials for Examples 1 - 8**

Materials

Bovine hides were obtained from the British Leather Centre (Northampton - GB) and ovine and caprine hides from LH Nichols Ltd (Yeovil - GB). Guinea pig liver 20 transglutaminase (GPL tTG) was obtained from Sigma-Aldrich Company Ltd and microbial transglutaminase (mTG) was purified from Activa WM (1% mTG, 99% maltodextrin) obtained from Ajinomoto Corp. using cation exchange chromatography. Biotin cadaverine (BTC) was obtained from Molecular Probes Inc. and all other chemicals were obtained from Sigma-Aldrich Company Ltd.

25

Purification of mTG from Activa WM by cation exchange chromatography

Activa WM (50g) was dissolved in 400ml of ice-cold 20mM phosphate buffer, 2mM EDTA pH 6.0 and filtered through Whatman No 1 filter paper to remove undissolved solids. The solution was diluted to 2l with ice cold 20mM phosphate buffer, 2mM 30 EDTA pH 6.0 and loaded onto a 100ml SP-Sepharose FF column overnight at a flow rate of 5mlmin⁻¹ by recycling. The column was washed with 200ml of ice cold 20mM phosphate buffer, 2mM EDTA pH 6.0 and proteins were eluted with a 0-1000mM gradient of NaCl in 20mM phosphate buffer, 2mM EDTA pH 6.0 over 80min, collecting 5ml fractions. Fractions were assayed for protein using the BioRad DC 35 protein assay (BioRad). Fractions containing mTG were pooled, aliquoted, freeze dried and stored at -70°C.

Biotin labelling of proteins

Microbial transglutaminase (1mgml^{-1}), affinity purified GPL tTG (1mgml^{-1}), and pancreatic trypsin (1mgml^{-1}) were dialysed against 0.1M NaHCO_3 pH 9 overnight at 4°C . After dialysis, a solution of 1mgml^{-1} biotinamidocaproate N-hydroxysuccinimide ester in dimethylsulphoxide (DMSO) was added to each enzyme solution in the ratio 5 1:8. Labelling reactions were incubated overnight at 4°C with gentle agitation. The reactions were then dialysed against 50mM Tris HCl , 1mM EDTA pH7.5 overnight at 4°C to remove unincorporated label and DMSO. Labelled proteins were analysed by 10 SDS-PAGE and a blotted gel was used to assess the biotin incorporation by reacting with an avidin-peroxidase conjugate and staining with $3,3'$ diaminobenzidine (SigmaFast DAB peroxidase substrate).

Uptake of proteins

Reaction mixes for the rehydration of the freeze-dried samples were prepared on ice 15 as follows. For mTG: 50mM Tris HCl pH 7.5, 0.5mgml^{-1} biotinylated mTG. For GPL tTG: 50mM Tris HCl pH 7.5, 2mM EDTA , $4\text{mM dithiothreitol}$, 0.5mgml^{-1} biotinylated GPL tTG. Reaction mixes (2ml per hide section) were added to samples of freeze-dried hide and shaken vigorously on a platform orbital shaker at room 20 temperature for 2 hour to allow uptake. Calcium chloride was added to the GPL tTG sample (to 5mM) and the samples were then incubated with shaking for a further two hours at 37°C . A companion experiment was set up identically except that native enzymes (ie not biotinylated) were used and $0.5\text{mM biotin cadaverine}$ was added to the reaction mixtures prior to soaking. Incorporation of BTC by transglutaminases 25 would then occur at the limits of penetration of the enzymes and would also confirm that enzyme activity was maintained within the hide.

The biotinylated enzyme treated hide samples were fixed with 4% neutral buffered formalin to ensure that the biotinylated transglutaminases could not diffuse during 30 subsequent processing. Samples treated with enzyme and biotin cadaverine were washed overnight at 4°C in 50mM Tris HCl pH 7.5, 2mM EDTA , with shaking, to ensure removal of unincorporated biotin cadaverine. Samples were then frozen and $20\mu\text{m}$ sections were cut using a cryostat.

Detection of biotin in hide sections

35 Sections were washed in phosphate buffered saline (PBS) to remove cryostat mounting medium and salts. The sections were incubated in blocking solution (1%

bovine serum albumin in PBS) for 60 min at 37°C and washed again with PBS. Cy5-streptavidin conjugate (2 μ gml⁻¹ in blocking solution) was added to each section and incubated overnight at 4°C. Sections were washed 3 times with PBS and mounted using a fluorescence mounting medium. The samples were examined by fluorescent confocal microscopy using laser excitation at 633nm and detection of emitted light through a 665nm long pass filter.

Enzymatic digestion of protein for ϵ (γ -glutamyl) lysine crosslink analysis

Samples of protein (approximately 10mg) were precipitated by the addition of 100% trichloroacetic acid to a final concentration of 10% and incubated on ice for 10min. Protein was collected by centrifugation at 13000g for 5min at room temperature. The supernatant was discarded and the pellet thoroughly resuspended in 250 μ l of 10% TCA. Protein was collected by centrifugation as before and washed three times with 250 μ l of diethyl ether/ethanol (1:1), ensuring complete dispersion of the pellet. The pellet was then washed in the same way three times with diethyl ether, and dried at room temperature for 30min. The pellet was rehydrated in 50 μ l of water and sonicated briefly to aid dispersion. To this was added 950 μ l of 0.1M ammonium bicarbonate pH 8.0 and 1 crystal of thymol to prevent bacterial growth during subsequent incubations. Proteolytic treatments were then performed in the following way: 10 μ l of 0.5M CaCl₂ and 10 μ l of collagenase (Clostridiopeptidase A) (10mgml⁻¹ in 0.1M NH₄HCO₃) were added and incubated at 32°C for 16hr with shaking; 10 μ l of subtilisin carlsberg (10mgml⁻¹ in 0.1M NH₄HCO₃) was added and incubated at 32°C for 16hr with shaking. The subtilisin digestion was repeated twice more. A protein assay was performed at this point to give an indication of protein concentration for subsequent calculations. 10 μ l of pronase (15mgml⁻¹ in 0.1M NH₄HCO₃) was added and incubated at 32°C for 16hr with shaking. This digestion was repeated once. Proteases were inactivated by heating to 100°C for 15min. Magnesium chloride was added to a final concentration of 5mM. Leucine aminopeptidase was activated by mixing the following components and incubating for 3hr at 37°C: 10 μ l manganese chloride (50mM), 90 μ l Tris HCL pH8.0 (10mM), 100 μ l leucine aminopeptidase (22.75u). Prolidase was activated by mixing the following and incubating at 37°C for 3hr: 20 μ l of manganese chloride (50mM), 80 μ l of Tris HCl pH8.0 (10mM), 80 μ l of distilled water, 20 μ l of prolidase (38.6U). 90 μ l of the activated leucine aminopeptidase and 75 μ l of activated prolidase was added and incubated at 37°C for 16hr with shaking. The leucine aminopeptidase and prolidase digestions were repeated once more. The pH of the sample was adjusted to between 6.75 and 7.0 with HCl and 10 μ l of

carboxypeptidase Y (20mgml⁻¹) was added and incubated at 30°C for 16hr with shaking. Samples were freeze dried and stored at -20°C.

Crosslink measurement by cation-exchange chromatography

5 Freeze dried samples of enzymatically digested hide samples were resuspended in 0.1M HCl and sonicated for 2min to aid dispersion. An aliquot (10-90µl) was mixed with loading buffer (0.2M lithium citrate, 0.1% phenol pH 2.2) and loaded onto a Dionex DC-4A resin column 0.5cm x 20cm using a Pharmacia Alpha Plus amino acid analyser. The buffer elution profile was as shown in the table below. Derivatisation
10 was performed post column using o-phthaldialdehyde (0.8M boric acid, 0.78M potassium hydroxide, 600mgml⁻¹ o-phthaldialdehyde, 0.5% (v/v) methanol, 0.75% (v/v) 2-mercaptoethanol, 0.35% (v/v) Brij 30) and absorbance was measured at 450nm. Dipeptide was determined by addition of known amounts of ε (γ-glutamyl)lysine to the sample and comparing peak areas.

15

TIME (min)	BUFFER	COLUMN
		TEMPERATURE
0-9	1	25°C
9-32	2	25°C
32-67	3	25°C
67-107	3	25°C
107-123	6	75°C
123-135	1	75°C
135-147	1	65°C
147-159	1	35°C
159-171	1	25°C

Buffer 1: 0.2M lithium citrate, 0.1% phenol, 1.5% (v/v) propan-2-ol pH 2.8.

Buffer 2: 0.3M lithium citrate, 0.1% phenol, 1.5% (v/v) propan-2-ol pH 3.0.

Buffer 3: 0.6M lithium citrate, 0.1% phenol pH 3.0.

20 Buffer 6: 0.3M lithium hydroxide.

Example 1

Bovine hides were subjected to a standard beam house processing sequence of soaking, liming and deliming. The deliming process was performed using sulphuric

acid instead of ammonium sulphate. The hides were then bated using 'Pancreol' bate. Bated bovine hide samples 5cm x 10cm were frozen to a temperature of -20°C and were freeze dried for 48hr under a vacuum of 6 mbar using an Edwards Modulyo freeze drier. 1.5 cm x 1.5cm samples were then cut using a hacksaw for uptake 5 analysis. Using similarly sized samples of wet bated bovine hide, prepared by wetting back freeze dried samples, wet and freeze-dried bated bovine hide samples were treated with GPL tTG, GPL tTG plus cadaverine, mTG; mTG plus cadaverine; and trypsin by soaking the samples in solution (see *uptake* above). The effectiveness of the uptake was assessed by biotinylation of the proteins (see *biotin labelling* above) 10 and the results (see *biotin detection* above) are shown in Figures 1 to 5. Additionally 1mm sections were cut from samples of mTG treated bated bovine hide for crosslink analysis by enzymatic digestion of protein followed by crosslink measurement by cation-exchange chromatography (Griffin et al (1982) *Anal Biochem.* 124 406-414). Figure 10 shows the distribution of crosslinks transversely across a section.

15

Example 2.

Bovine rawhide samples 5cm x 10cm were frozen to a temperature of -20°C and were freeze dried for 48hr under a vacuum of 6 mbar using an Edwards Modulyo freeze drier. 1.5 cm x 1.5cm samples were then cut using a hacksaw for uptake 20 analysis. Using similarly sized samples of wet bovine rawhide and freeze-dried bovine rawhide, samples were treated with trypsin, mTG and GPL tTG by soaking the samples in solution (see *uptake* above). The effectiveness of the uptake was assessed by biotinylation of the proteins (see *biotin labelling* above) and the results (see *biotin detection* above) are shown in Figure 6.

25

Example 3

Delimed bovine hide samples 5cm x 10cm were frozen to a temperature of -20°C and were freeze dried for 48hr under a vacuum of 6 mbar using an Edwards Modulyo freeze drier. 1.5 cm x 1.5cm samples were then cut using a hacksaw for uptake 30 analysis. Using similarly sized samples of wet delimed bovine hide and freeze-dried delimed bovine hide, samples were treated with trypsin, mTG and GPL tTG by soaking the samples in solution (see *uptake* above). The effectiveness of the uptake was assessed by biotinylation of the proteins (see *biotin labelling* above) and the results (see *biotin detection* above) are shown in Figure 7.

35

Example 4

Crust leather (air-dried leather) 1.5 cm x 1.5 cm samples were treated with mTG; GPL tTG, and mTG plus cadaverine by soaking the samples in solution (see *uptake* above). The effectiveness of the uptake was assessed by biotinylation of the proteins (see *biotin labelling* above) and the results (see *biotin detection* above) are shown in Figures 8 and 9.

5 **Example 5**

Pickled goats skin samples 5cm x 10cm were frozen to a temperature of -20°C and were freeze dried for 48hr under a vacuum of 6 mbar using an Edwards Modulyo freeze drier. 1.5 cm x 1.5cm samples were then cut using a hacksaw for uptake 10 analysis. Using similarly sized samples of wet pickled goats skin and freeze-dried pickled goats skin, samples were treated with GPL tTG and mTG by soaking the samples in solution (see *uptake* above). The effectiveness of the uptake was assessed by biotinylation of the proteins (see *biotin labelling* above) and the results (see *biotin detection* above) are shown in Figures 11 and 12.

15

Example 6

Wool-on slink skin samples 5cm x 10cm were frozen to a temperature of -20°C and were freeze dried for 48hr under a vacuum of 6 mbar using an Edwards Modulyo freeze drier. 1.5 cm x 1.5cm samples were then cut using a hacksaw for uptake 20 analysis. Using similarly sized samples of wet wool-on slink skin and freeze-dried wool-on slink skin, samples were treated with GPL tTG and mTG by soaking the samples in solution (see *uptake* above). The effectiveness of the uptake was assessed by biotinylation of the proteins (see *biotin labelling* above) and the results (see *biotin detection* above) are shown in Figures 13 and 14.

25

Example 7

Pieces of bated bovine hide were incubated in dry 100% acetone with shaking for 24hr, with changes of solvent at 8hr and 16hr to ensure maximal dehydration. The pieces of hide was then removed from the acetone and air dried for two hours at room 30 temperature. The dried samples were treated with mTG by soaking (see *uptake* above). The effectiveness of the uptake was assessed by biotinylation of the proteins (see *biotin labelling* above) and the results (see *biotin detection* above) are shown in Figure 15.

Results

Uptake of transglutaminases into bated bovine hide

The distribution of biotinylated GPL tTG after uptake for 2h into bated bovine hide
5 with or without freeze drying is shown in Figure 1. It can be seen that GPL tTG did
not penetrate wet bated bovine hide at all, being found as a thin layer on the grain and
flesh surfaces. After freeze drying, bated bovine hide allowed GPL tTG to penetrate
to a small degree throughout the section although the majority of the staining was
again found on the grain and flesh surfaces. The pattern of biotin cadaverine
10 incorporation into wet or freeze dried bated bovine hide confirmed that GPL tTG did
not penetrate the hide to any significant degree (Figure 2), with incorporation being
limited to a narrow layer on the grain and flesh surfaces. The only noticeable effect of
freeze drying on the uptake of GPL tTG was that biotinylated GPL tTG was found
deeper within the hair follicles, with corresponding increased biotin cadaverine
15 incorporation in these areas.

The pattern of uptake of biotinylated mTG into wet and freeze dried bated bovine hide
after 2h is shown in Figure 3, with the corresponding pattern of biotin cadaverine
incorporation shown in Figure 4. It can be seen that mTG penetrated wet bated bovine
20 hide to a depth of up to 1mm through both the grain and flesh sides, whereas mTG
fully penetrated freeze dried bated bovine hide in the same time. Biotin cadaverine
incorporation into wet bated bovine hide was limited to a depth of 0.5mm through the
grain side and 1mm through the flesh side, whereas fairly even biotin cadaverine
incorporation was observed throughout the freeze dried bated bovine hide, showing
25 complete penetration within 2h.

Figure 5 shows the penetration of biotinylated trypsin, mTG and GPL tTG into wet
and freeze dried bated bovine hide at 15min and 60min to assess the rate of uptake.
The smallest protein, trypsin (24kDa), fully penetrated freeze dried bated bovine hide
30 within 15min. GPL tTG (80kDa) penetrated poorly even after 60min, whereas mTG
(38kDa) penetrated almost fully after only 15min.

Uptake of enzymes into raw and delimed bovine hide

The depth of penetration of trypsin, mTG and GPL tTG into raw and delimed bovine
35 hide was determined by confocal microscopy as before (Figure 6). Freeze drying
enhances the uptake of trypsin, mTG and GPL tTG into rawhide, although the

increase in penetration is small, size dependent and only through the flesh side. There was no enhancement of uptake by freeze drying through the grain side.

5 Penetration of trypsin, mTG and GPL tTG into wet and freeze dried delimed bovine hide was poor (Figure 7), although mTG appeared to penetrate better into freeze dried delimed hide than either trypsin or GPL tTG.

Uptake of transglutaminases into bovine crust leather

10 The depth of penetration of mTG and GPL tTG after soaking into bovine crust leather after 60min was determined. Figure 8 shows that biotinylated GPL tTG did not penetrate through the grain side at all, but penetrated to a depth of about 0.75mm through the flesh side. No biotinylated GPL tTG could be detected within the fibre bundles due to exclusion on the basis of size. Biotinylated mTG penetrated to a depth of 0.25mm through the grain and 1.75mm through the flesh side, leaving a narrow 15 0.5mm gap in between. The pattern of biotin cadaverine incorporation after uptake of mTG and GPL tTG into bovine crust leather is shown in Figure 9. A similar pattern of incorporation was observed compared to the localisation of biotinylated transglutaminases.

20 ***Distribution of $\epsilon(\gamma\text{-glutamyl})$ lysine throughout bated bovine hide***

The distribution of $\epsilon(\gamma\text{-glutamyl})$ lysine incorporated by mTG after uptake into freeze dried bated bovine hide was determined by performing crosslink analysis on 1mm thick layers in the grain, corium and flesh side. Figure 10 shows the amount of crosslink in these areas compared to the pattern of biotin cadaverine incorporation 25 from a separate experiment under identical conditions. The amount of $\epsilon(\gamma\text{-glutamyl})$ lysine incorporated was found to be fairly even across the entire section, with about 30% more found in the grain.

Uptake of transglutaminases into pickled goat skin

30 The degree of penetration of biotinylated GPL tTG and mTG into wet and freeze dried pickled goat skin was determined (Figures 11 & 12). Biotinylated GPL tTG did not penetrate wet pickled goat skin except for some staining in the hair follicles. Penetration was good with freeze dried pickled goat skin, staining being found 35 throughout the section. Biotinylated mTG showed better penetration into wet pickled goat skin than GPL tTG and showed excellent uptake throughout the whole section with freeze dried skin.

Uptake of transglutaminases into pickled wool-on slink skins

The degree of penetration of biotinylated GPL tTG and mTG into wet and freeze dried pickled wool-on slink skin was determined (Figures 13 & 14). Biotinylated GPL tTG did not penetrate wet or freeze dried pickled wool-on slink skin at all, although staining was found in the hair follicles in each case. Biotinylated mTG also showed poor penetration of wet pickled wool-on slink skin. However, freeze drying enhanced uptake through both the grain and flesh sides, with almost complete penetration in 60min.

10 Uptake of transglutaminases into acetone dried bated bovine hide

The degree of uptake of biotinylated mTG into acetone dried bated bovine hide is shown in Figure 15. After 60min, enzyme was found evenly throughout the cross-section.

15 Example 8

Freeze dried bated leather samples of approximately 1/4 of A4 size were treated in a range of enzyme reaction solutions, containing one or more of mTG (ACTIVA WM from Ajinomoto Inc), keratin (ATIFAX WOK from Dr. Tho. Böhme Kg) or putrescine (from Sigma), at 4 °C until complete wet back was achieved. The offers assayed (expressed as w/w-hide %) were: 500 % Tris (hydroxymethyl) methyl amine HCl pH 7.4 solution; 0.05 % mTG ; 10 % keratin ; 0.23% putrescine.

20 After wetting back, the treatment was continued for a further 2 hours at 37 °C to allow for enzyme incorporation of the keratin and/or putrescine into the leather matrix. The samples were then separately processed and then dyed with an acid dye. The lightness 25 of the leathers was measured as an indicator of the strength of colour and the samples were then assayed for dye release in an artificial perspiration solution. The data are shown in Figures 16 and 17.

With the exception of putrescine treatment, all the treatments increased the depth of 30 shade of dyeing (measured as reduced lightness of shade) on the grain surface of the bated leather, compared to the control (Figure 16). A similar pattern was observed on the flesh surface of the samples although the greatest increase in the depth of shade was achieved after treatment with TG alone (Figure 17). ANOVA (analysis of variance) of the dye release by perspiration solution showed a significant difference 35 between the treatment groups with trend for increasing dye release with increasing degree of treatment when compared to the control ($F = 7.47, p = <0.0001$).

Example 9

This example uses crust leather samples of approximately 1/4 of A4 size and the solutions previously described in Example 8. The lightness of the leathers was measured as an indicator of the strength of colour and assayed for dye release in an artificial perspiration solution. The data are shown in Figures 18 and 19. Treatment with putrescine, putrescine + keratin, TG + keratin, or TG + keratin + putrescine increased the depth of shade on the grain surface compared to the control, with the greatest increase measured in the sample treated with TG + keratin (Figure 18). Similarly, treatment with putrescine + keratin, TG, TG + keratin, or TG + keratin + putrescine increased the depth of shade on the flesh surface of the samples (Figure 18). As for the grain surface, the greatest increase in depth of shade was measured after treatment with TG + keratin. ANOVA analysis of the dye release by perspiration solution showed a significant difference between the treatment groups ($F = 8.16, p = < 0.0001$). However, in contrast to the bated samples, treatment with TG or TG + keratin was found to significantly decrease the dye release in the perspiration solution ($p = 0.0027$ and 0.0002 , respectively). The data showed that pre-treatment of crust leather with TG and/or keratin could increase the depth of colour of dyeing with an acid dye and also improved dye fastness in artificial perspiration solution.

Example 10

The procedures of Example 9 were then repeated using A4 size crust leather samples. The samples were treated with TG, keratin or TG + keratin and then dyed with either an acid or a reactive dye. After dyeing, the lightness of the leather was measured. The samples were then treated with an artificial perspiration solution or a wash-fastness solution. After treatment with these solutions, the lightness of the leather was remeasured in order to assess the extent of dye loss relative to a conventionally dyed control sample. The results are shown in Figures 20 - 23.

All three treatments improved the depth of shade, relative to the control, of dyeing with an acid dye (Figure 20 and 21). Treatment with TG + keratin resulted in the largest increase in depth of shade. After treatment with an artificial perspiration solution, the relative depth of shade of the samples treated with TG or TG + keratin, had increased relative to the control, indicating that less dye had been lost by TG-treated samples than by the control. The depth of shade of the sample treated with keratin only, fell relative to the control after treatment with artificial perspiration solution, but remained a darker shade than the control sample. A similar pattern was

observed after treatment of the samples with a wash-fastness solution. All three treatments increased the relative depth of shade of the leather, with the largest increase observed after treatment with TG + keratin. The change in relative depth of shade in the treated samples was larger than that observed after treatment with the

5 artificial perspiration solution. This suggests that the wash-fastness solution was more efficient at removing dye from the control sample than the artificial perspiration solution, but that treatment with TG, keratin or TG + keratin could reduce or prevent dye loss in the wash-fastness solution.

10 Similar results were observed for the samples dyed with the reactive dye. Treatment with TG appeared to have decreased the depth of shade prior to treatment with the artificial perspiration solution (Figure 22). This is thought to be a process artefact, arising from the small scale at which the process was carried out, as the depth of shade after treatment with TG was increased prior to treatment with the wash-fastness

15 solution (Figure 23). Treatment with the wash-fastness solution reduced the relative depth of shade of the samples treated with TG or keratin, relative to the control, albeit that the depth of shade was still greater than the control. However, as for samples dyed with the acid dye, pre-treatment with TG + keratin resulted in an increased depth of shade relative to the control, after treatment with wash-fastness solution,

20 suggesting that the treatment had increased the dye fastness.

The data show that treatment of crust leather with TG and/or a biopolymer, such as keratin, will increase the efficiency of dyeing, as indicated by the increase in depth of shade of the leather, and will increase the resistance of the leather to dye loss in

25 artificial perspiration solution or wash-fastness solution. The greatest effects were observed after treatment with TG + keratin, suggesting that the hypothesis, that enzyme incorporation of a biopolymer into the collagen matrix of leather will enhance the uptake of dyes by increasing the number dye binding sites, is valid.

30 **Example 11**

Crust wool on sheepskin samples (chrome tanned and fat-liquored) were treated over 2 hours at 40 °C with mTG/Keratin and subsequently dyed in a conventional acid dye process. The resistance of wool to abrasion of the leather was measured. The protocol was carried out as follows:

35

A circular test specimen was taken from the sheep leather sample. The test piece was

weighed and placed in a specimen holder with the wool facing downwards. The test specimen was rubbed against an abrasive medium under an applied pressure of 12 KPa for 1000 cycles. Once the test was completed, the weight of the samples was measured again. The resistance of wool to abrasion was expressed as the relative loss 5 in weight.

As shown in Figure 24, evaluation of the effect of mTG/keratin treatments on wool resistance to abrasion suggest that increasing the mTG offer results in a wool more resistant to abrasion. This is true at 0%, 10% and 20 % keratin offer. However, that 10 was not observed at 5% keratin offer. The best results were achieved at 10 % keratin and 0.1% mTG.

Example 12

Raw hide was processed as follows:

15 (a) by traditional calcium hydroxide/sodium sulphide liming with subsequent ammonium sulphate deliming,
(b) by sodium hydroxide/sodium sulphide liming with subsequent acid sulfuric deliming,
(c) by dispase (protease) unhairing (at a neutral pH).

20 The calcium hydroxide/sodium sulphide limed and sodium hydroxide/sodium sulphide limed hides were further treated with a bathing enzyme. The dispase hide did not undergo the enzyme bathing process, as dispase is a neutral protease enzyme which should have already provided the bathing effect on the hide.

25 After three washes with water, the samples were frozen in liquid nitrogen, freeze dried for 72 h and finely ground. 0.1 g of the hide powder from the three procedures (traditional liming, sodium hydroxide/sodium sulphide liming and dispase unhairing) was treated with 0.1% mTG w/dry-weight hide for 2 hours at 37 °C in a tris HCl 30 buffer solution pH 7.4 (controls with no mTG were also carried out for each sample). After the treatment, the samples were frozen in liquid nitrogen and freeze dried overnight. 50 mg of each sample was treated with 100 µg of collagenase from *Clostridium histolyticum* in 10 ml of Tris HCl buffer solution pH 7.4 5 mM CaCl₂. The collagenase digestion was carried out overnight at 37 °C. 5ml of the solution 35 were hydrolysed overnight in 5ml of 6M HCl at 100 °C and the hydroxyproline content analysed by the Chloramine T method

The results shown in Figure 25 provide evidence that mTG mediated crosslinking results in a hide more resistant towards microbial collagenase. This is true for the three substrates investigated. The most significant difference is observed in the samples that underwent the NaOH/Na₂S liming, as the amount of hydroxyproline in 5 the untreated control is nearly twice as high as the mTG treated sample (1351 micromol/l for the mTG treated sample, against 2262 micromol/l for the untreated control). This correlates well with other tests carried out by some of the present inventors which demonstrated that NaOH/Na₂S liming with a subsequent H₂SO₄ deliming would provide a better mTG substrate than traditional liming. It is evident 10 that the dispase limed samples (mTG treated and control) were more resistant towards microbial collagenase than the other two groups. There is a possibility that residual dispase present in the hide powder could be attacking the collagenase with the consequent inhibition of the collagenase activity.

15 **Industrial Applicability**

The process of this invention represents a cleaner technology for the leather industry, and when used as an adjunct of a dyeing process allows reduced dye offers, lower dye effluent loading and better performance characteristics of the dyed leather.

CLAIMS

1. A process for the treatment of leather in preparation for dyeing, in which leather or animal skins are treated with a transglutaminase and optionally a polymer or oligomer containing glutamine and/or lysine.
5
2. A process according to claim 1 in which the transglutaminase is a microbial transglutaminase.
- 10 3. A process according to claim 1 or 2 in which the polymer is a biopolymer.
4. A process according to claim 3 in which the biopolymer is keratin or casein.
- 15 5. A process according to any one of claims 1 to 4 in which the leather or animal skins are treated with a transglutaminase, a glutamine and/or lysine containing polymer or oligomer and a polyamine.
6. A process according to claim 5 in which the polyamine is putrescine or cadaverine.
20
7. A process for dyeing leather comprising treating leather by a process as claimed in any one of claims 1 to 6 and applying a dyestuff to the pre-treated leather.
- 25 8. Use of a transglutaminase and optionally a polymer or oligomer containing glutamine and/or lysine in a process for dyeing animal skins or leather, to improve dyeing quality
9. Use of a transglutaminase and optionally a polymer or oligomer containing glutamine and/or lysine when processing animal skins or leather, to improve abrasion resistance.
30
10. Use of a transglutaminase and optionally a polymer or oligomer containing glutamine and/or lysine when processing animal skins or leather, to improve collagenase resistance

11. A process for the treatment of animal hides, skins or leather in which the hide, skin or leather is treated with a solution of protein or enzyme so that the protein or enzyme is absorbed into the hide, skin or leather characterised in that the hide or leather is dried prior to treatment with the protein or enzyme, and the solution of protein or enzyme is applied to the dried hide, skin or leather.
5
12. A process according to claims 11, in which the leather or animal hide or skin is dried by a method selected from air drying, freeze drying, oven drying, vacuum oven drying and solvent dehydration.
10
13. A process according to claim 11 or 12 in which the enzyme belongs to the class of transferases, hydrolases, lysases or isomerases.
15
14. A process according to claim 11 or 12 in which the enzyme is a transglutaminase.
15
15. A process according to claim 14 in which the transglutaminase is used together with a protein or polyamine to bind the protein or polyamine into the collagen matrix.
20
16. A process according to claim 11 or 12 in which the enzyme is a protease such as trypsin.
25
17. A process according to any one of claims 11 to 16 in which the animal hide is rawhide or skin.
25
18. A process according to any one of claims 11 to 16 in which the animal hide is a bated hide or skin.
30
19. A process according to any one of claims 11 to 16 in which the solution of protein or enzyme is applied to crust leather.

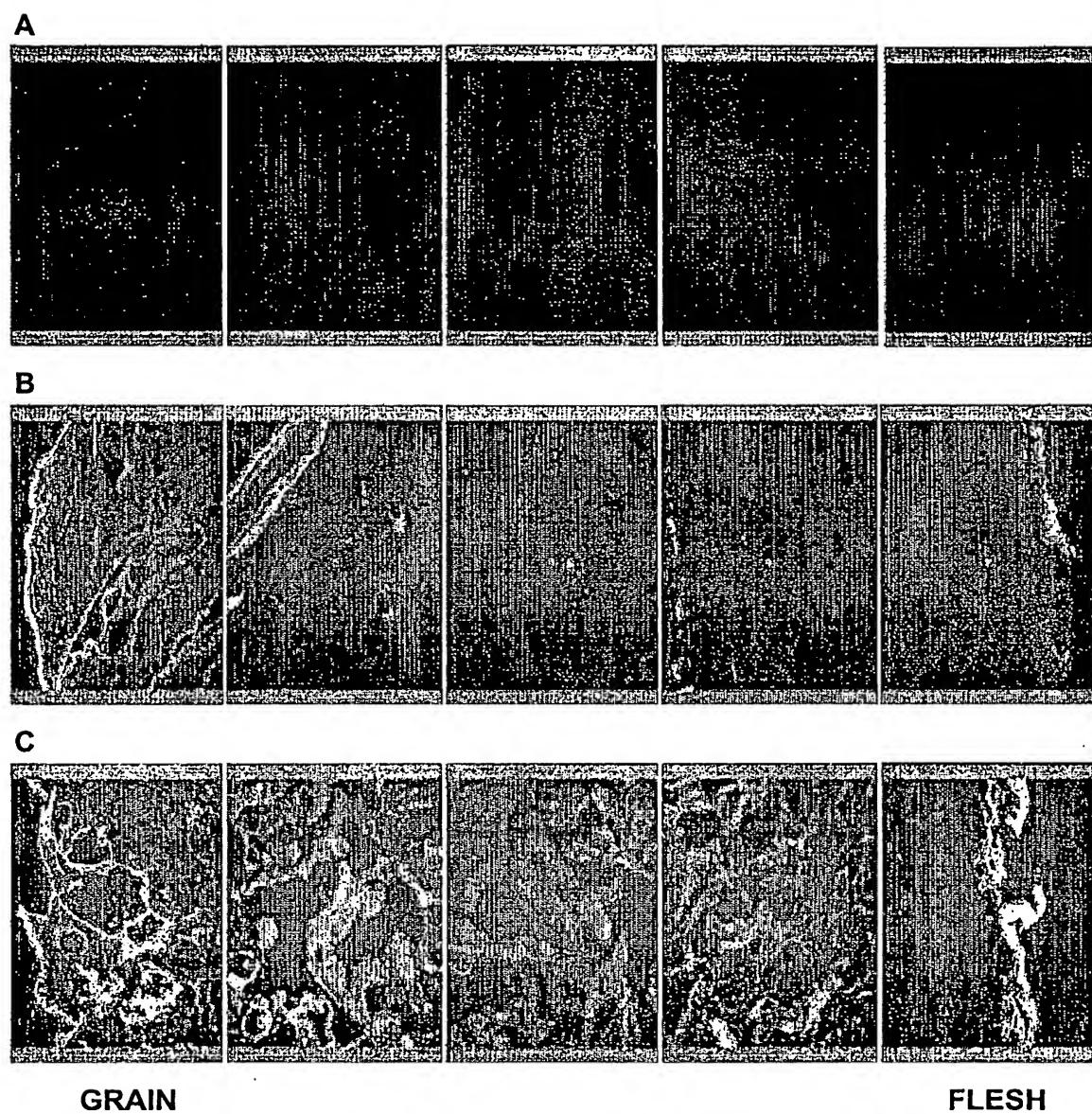
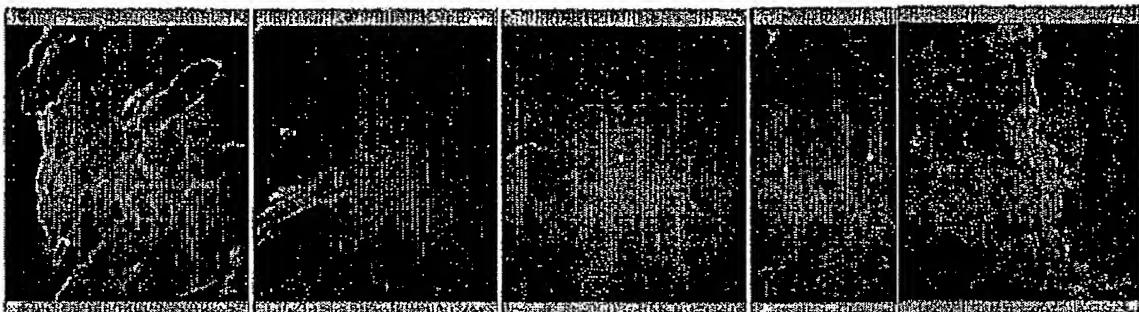
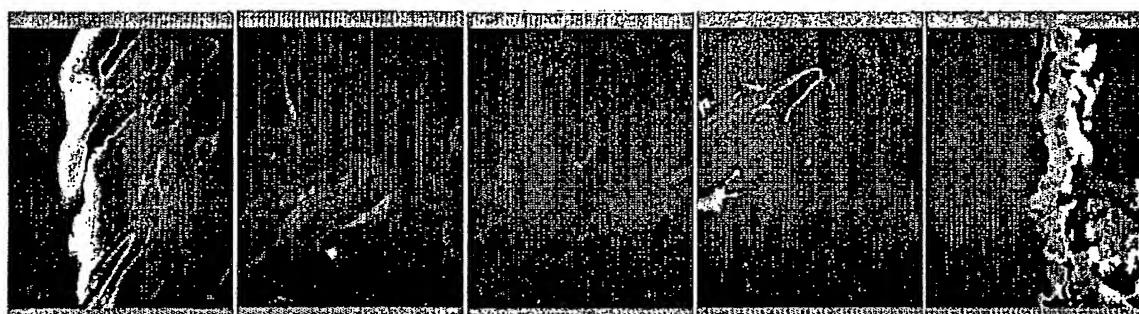
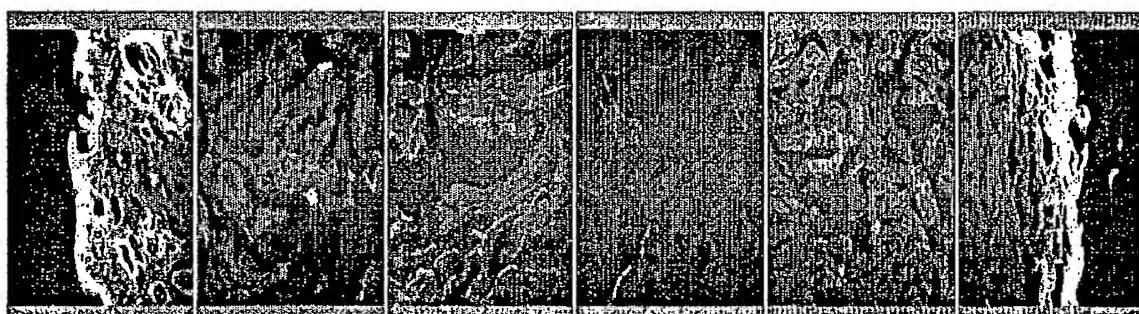
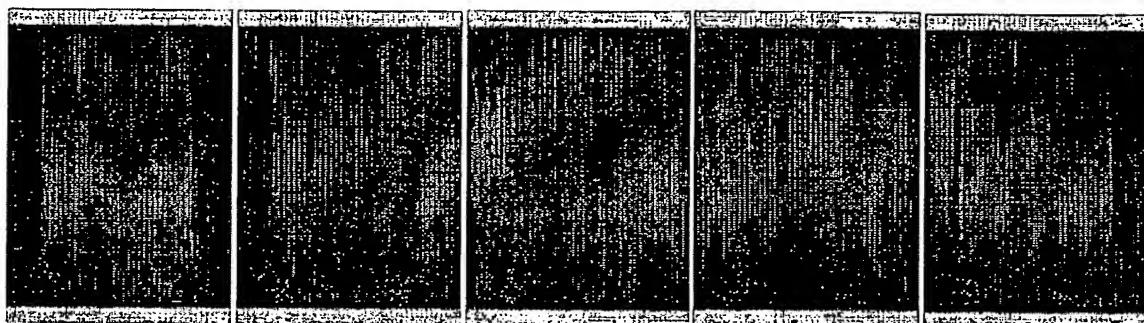
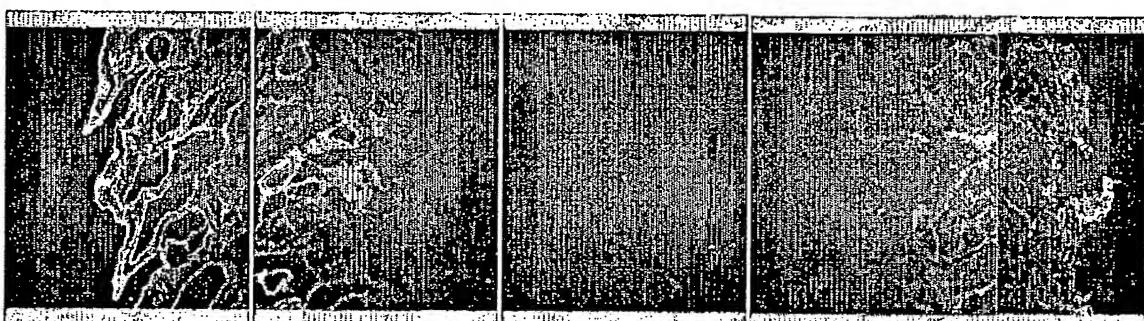


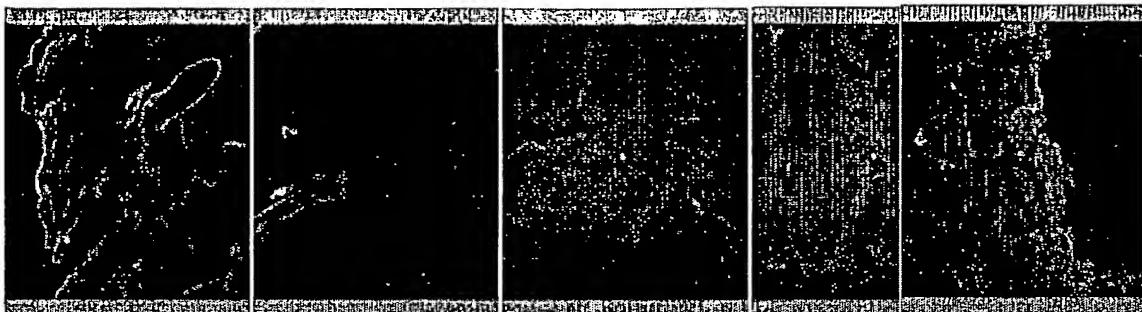
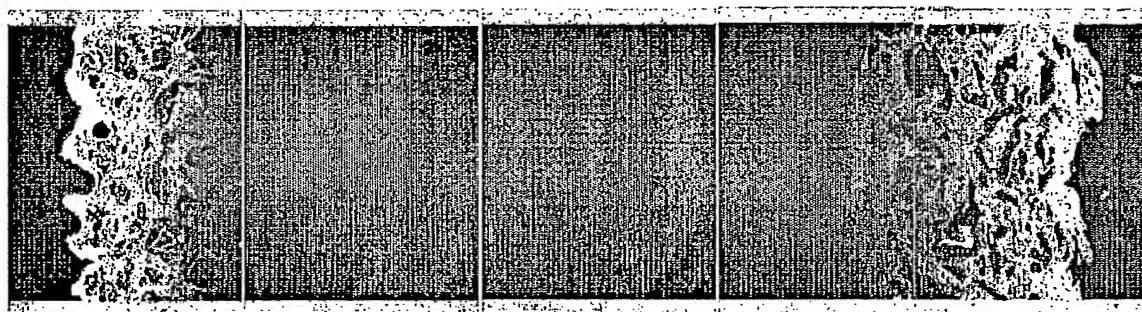
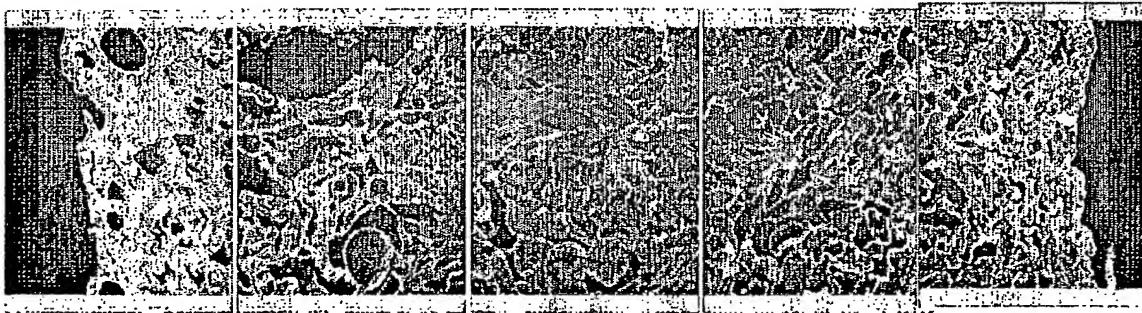
Fig. 1

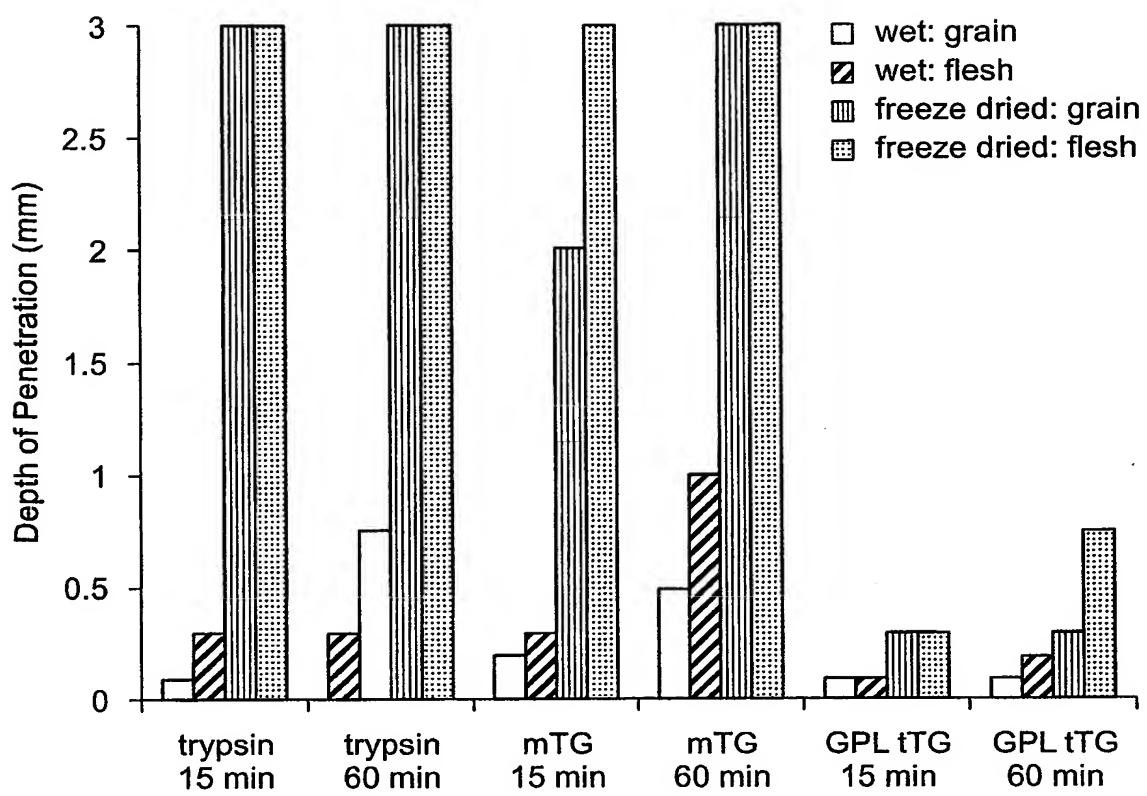
A**B****C****Fig. 2**

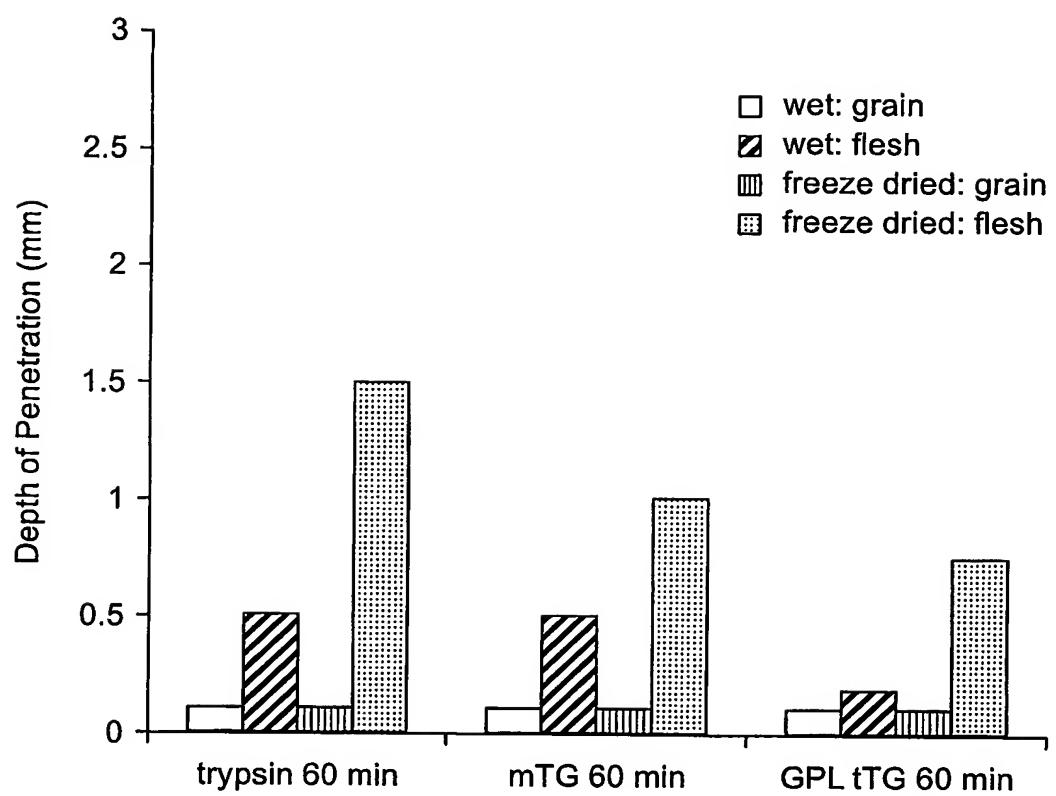
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A**B****C****GRAIN****FLESH****Fig. 3**

4/23

A**B****C****Fig. 4**

**Fig. 5**

6/23**Fig. 6**

7/23

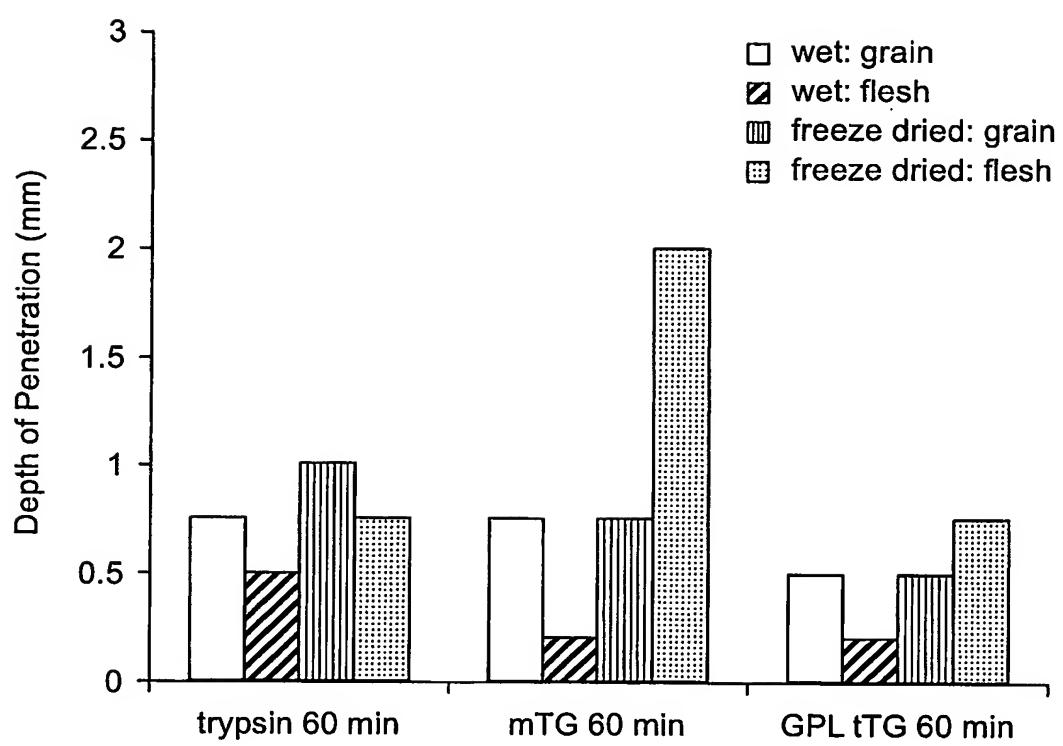
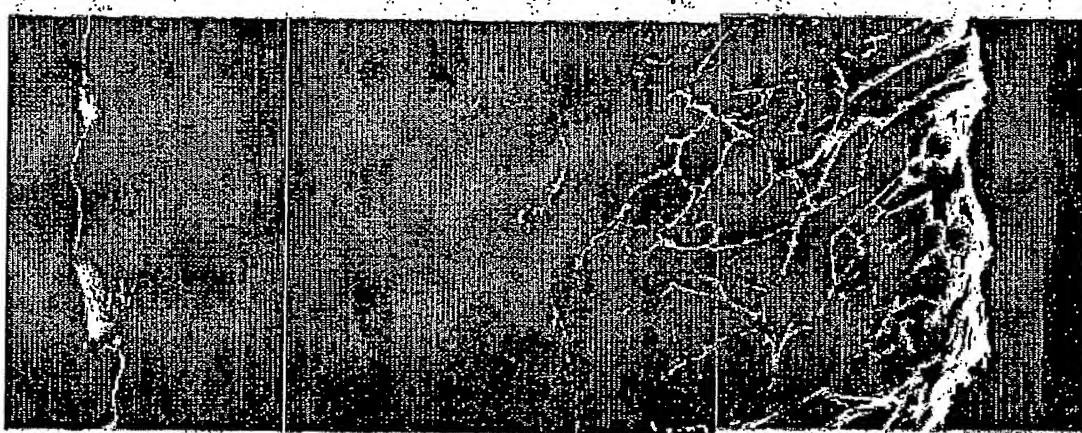


Fig. 7

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A



B

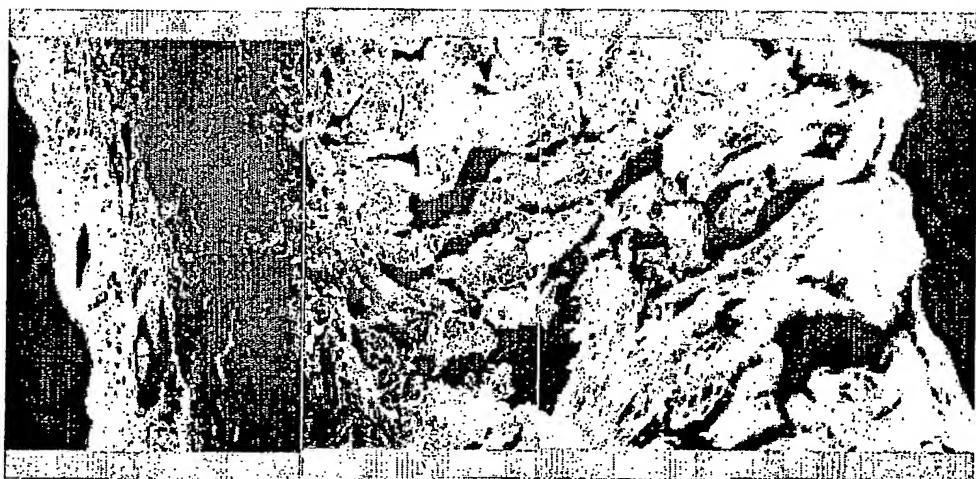
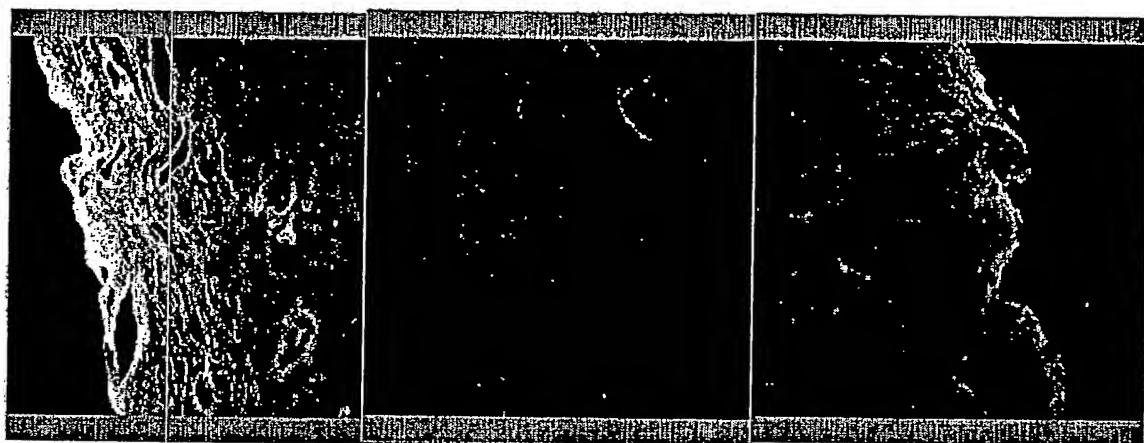
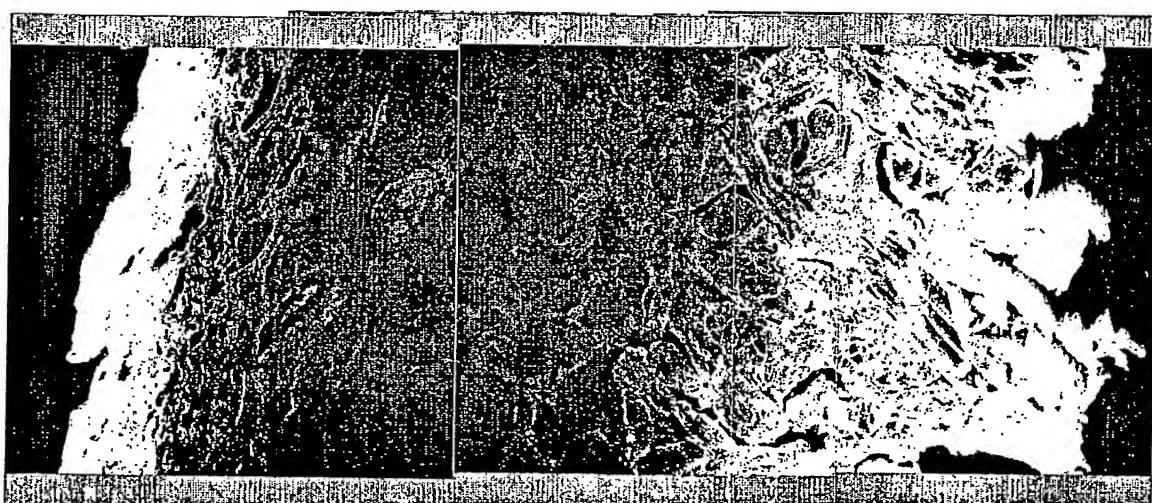


Fig. 8

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A**B****GRAIN****FLESH****Fig. 9**

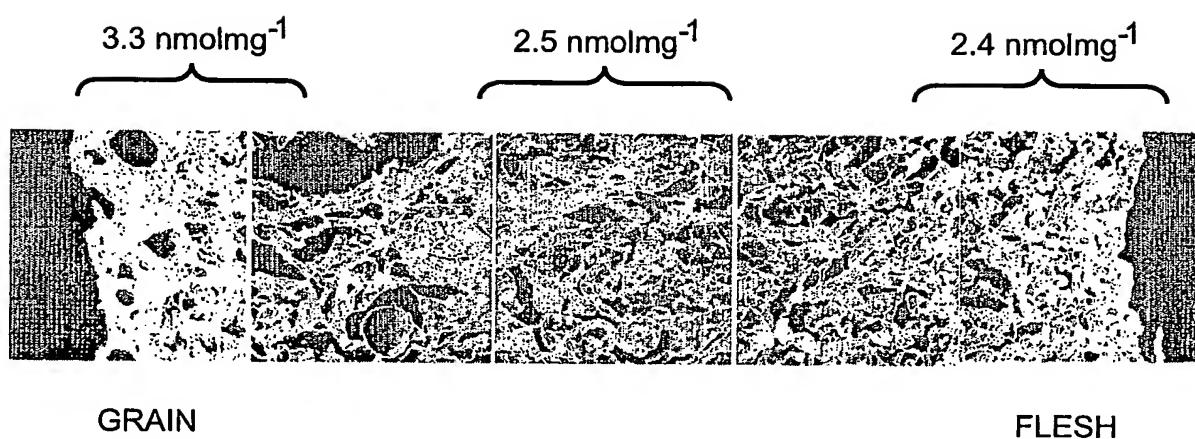
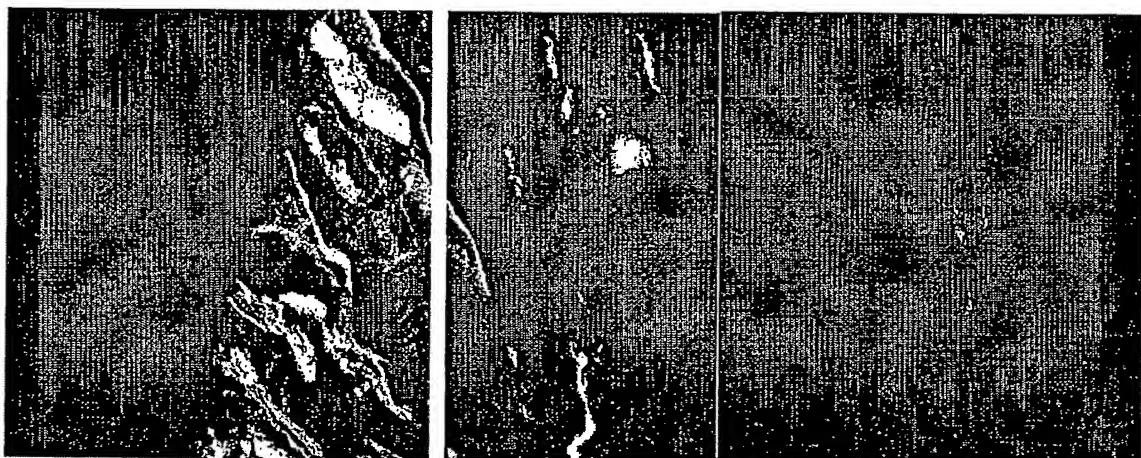
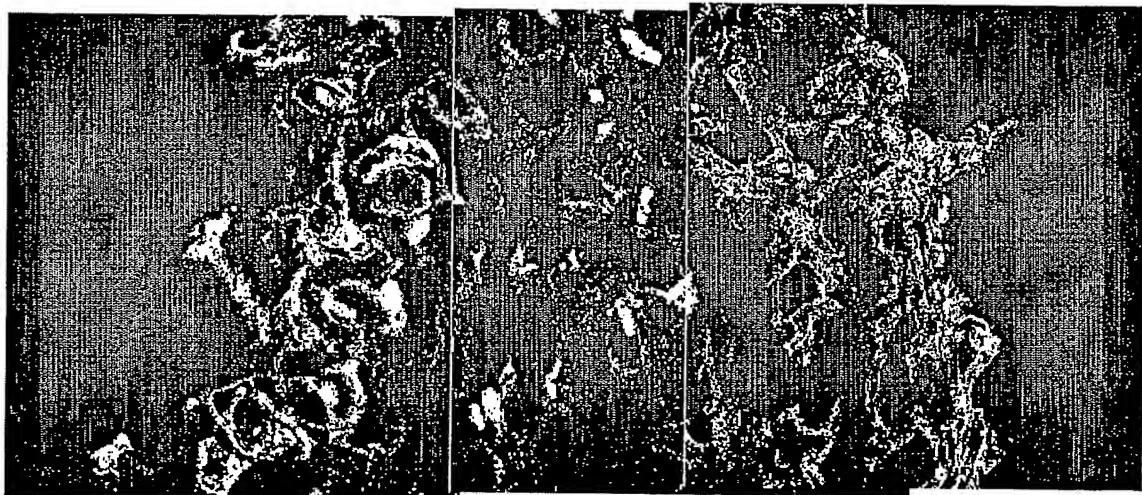
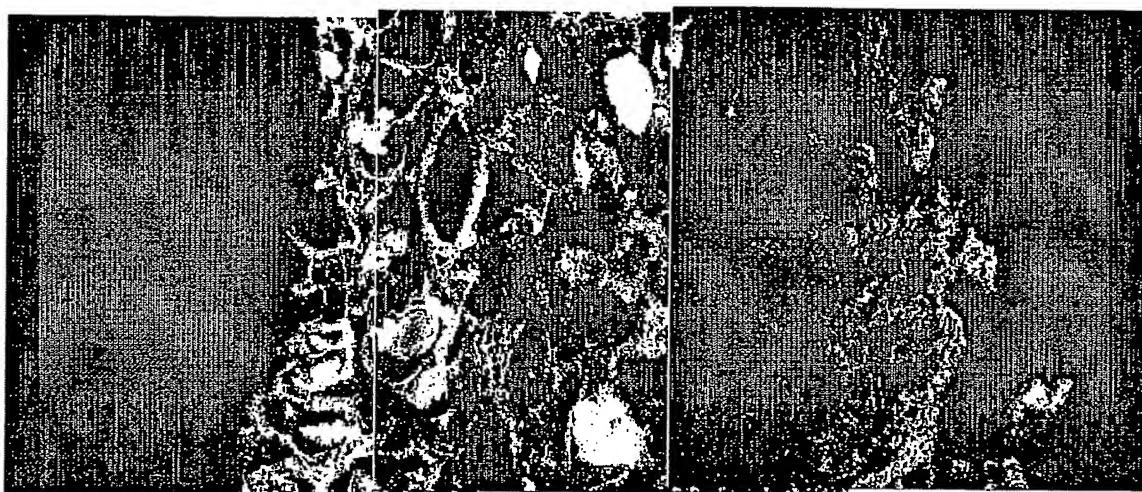


Fig. 10

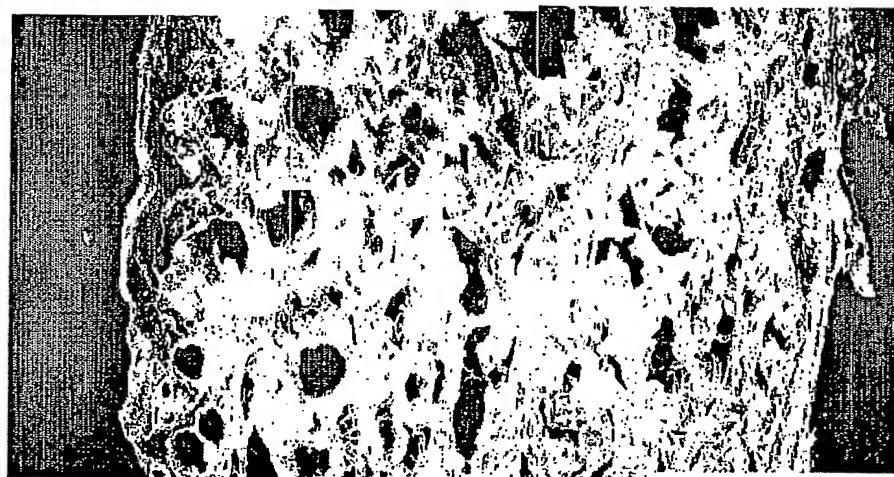
11/23**A****B****GRAIN****FLESH****Fig. 11**

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A



B



GRAIN

FLESH

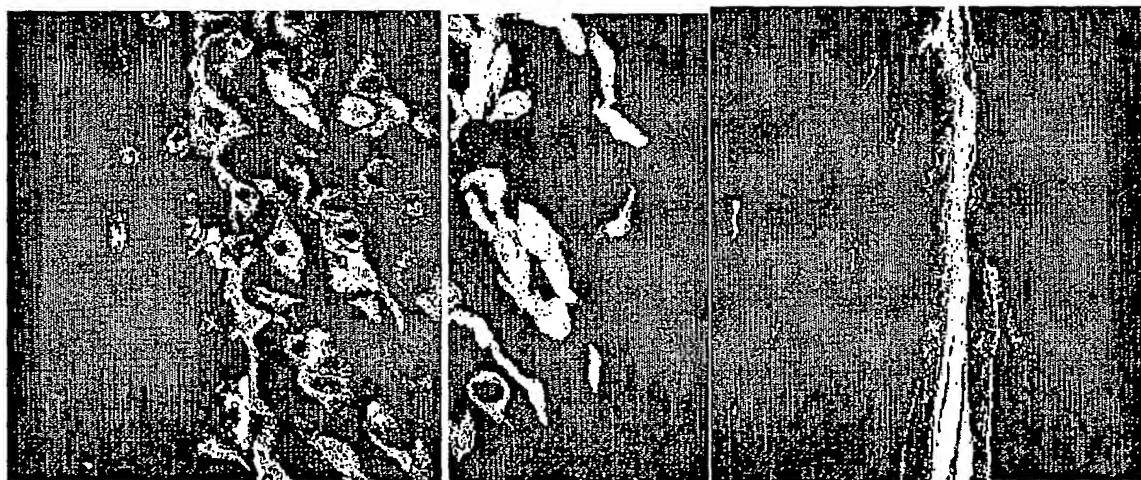
Fig. 12

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A



B



GRAIN

FLESH

Fig. 13

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A



B



GRAIN

FLESH

Fig. 14

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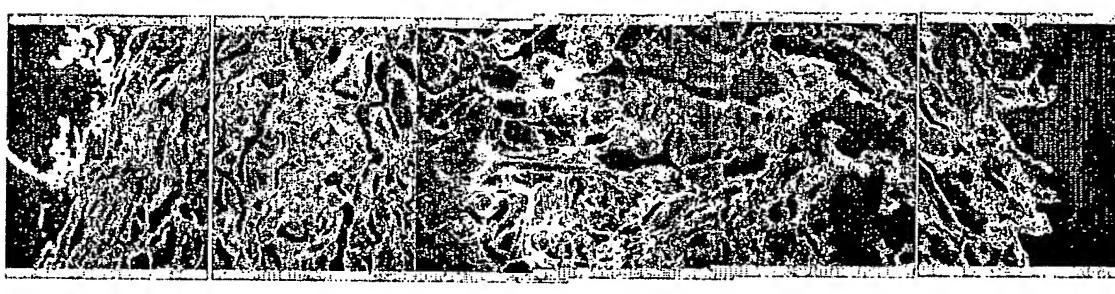


Fig. 15

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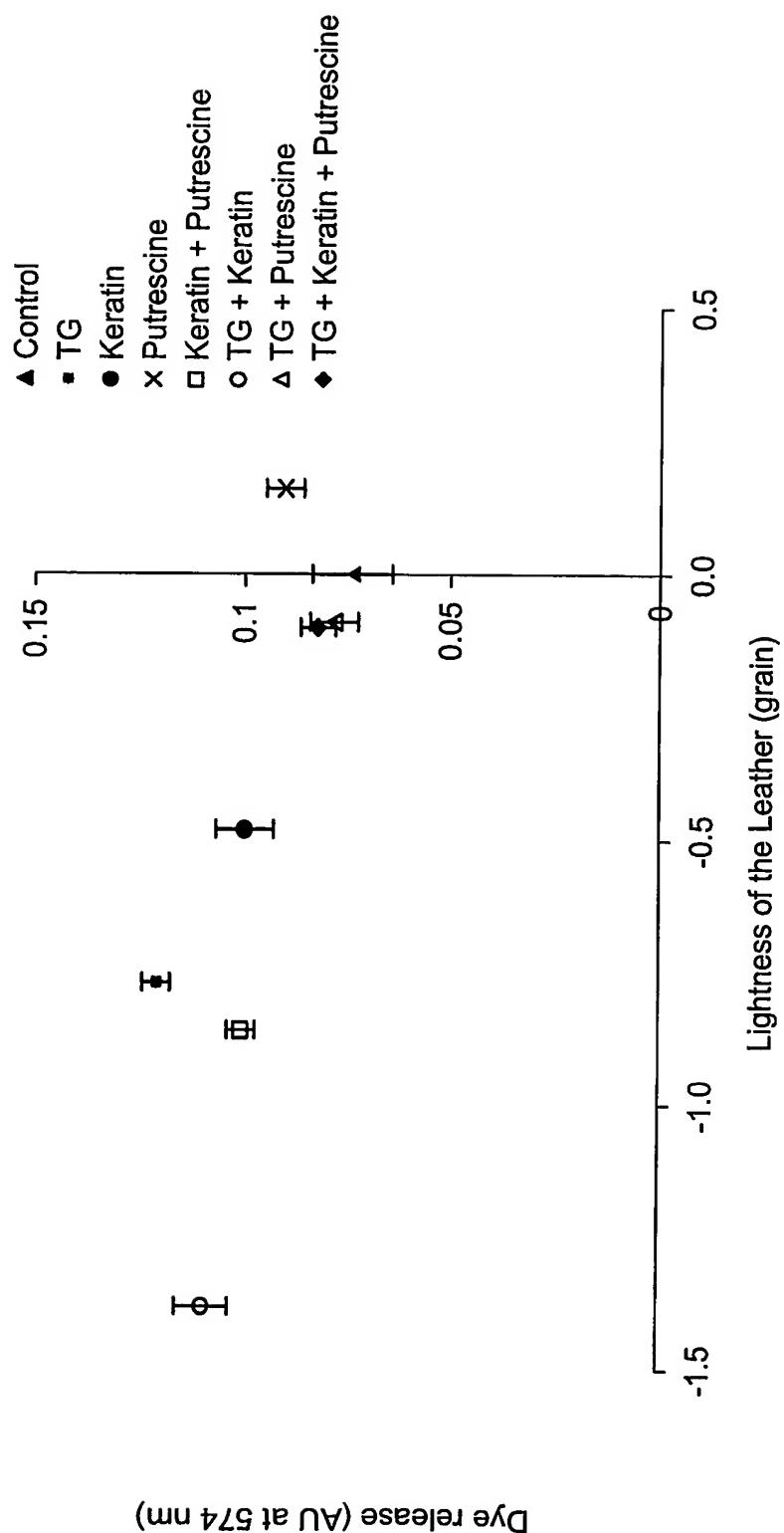


Fig. 16

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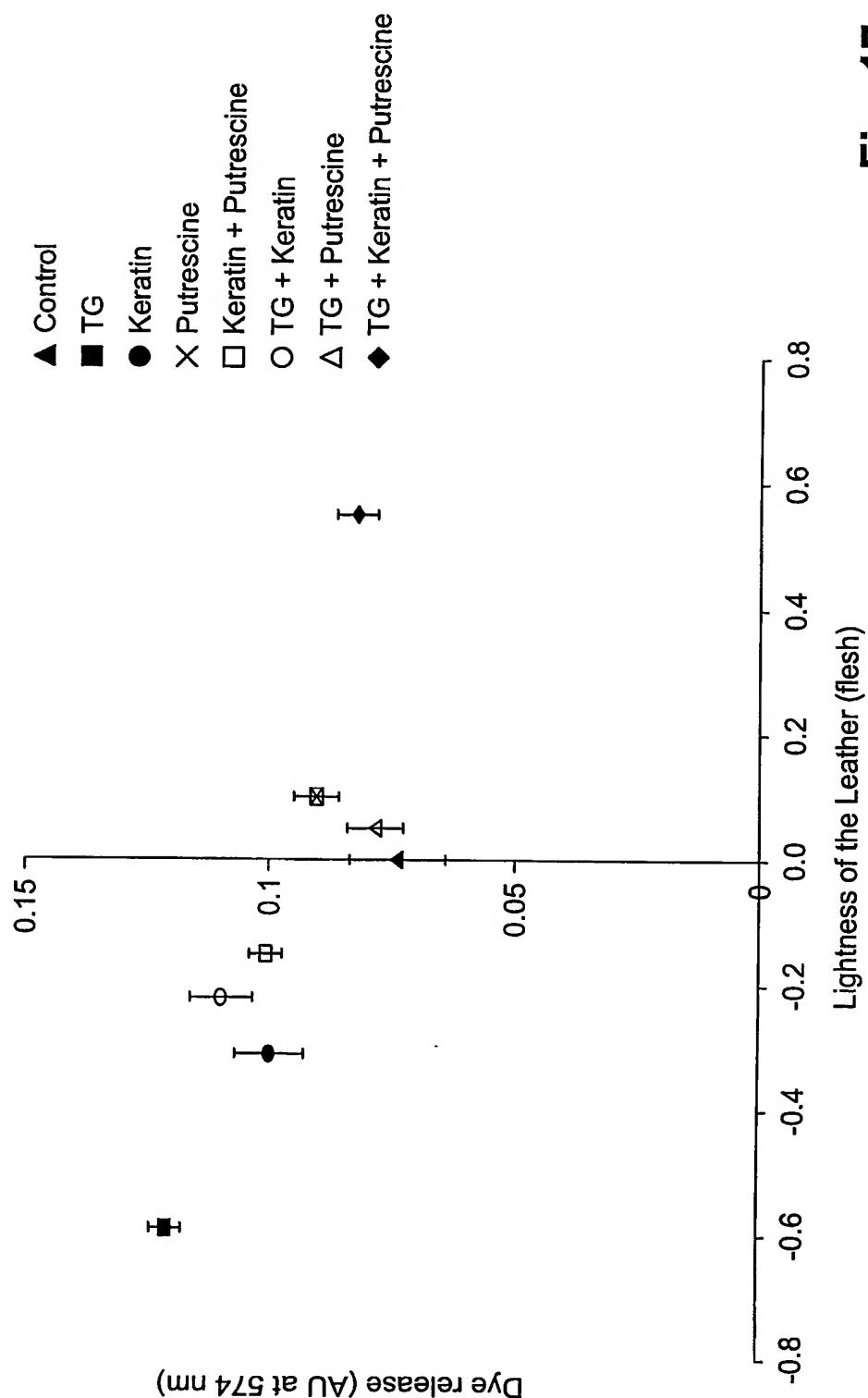


Fig. 17

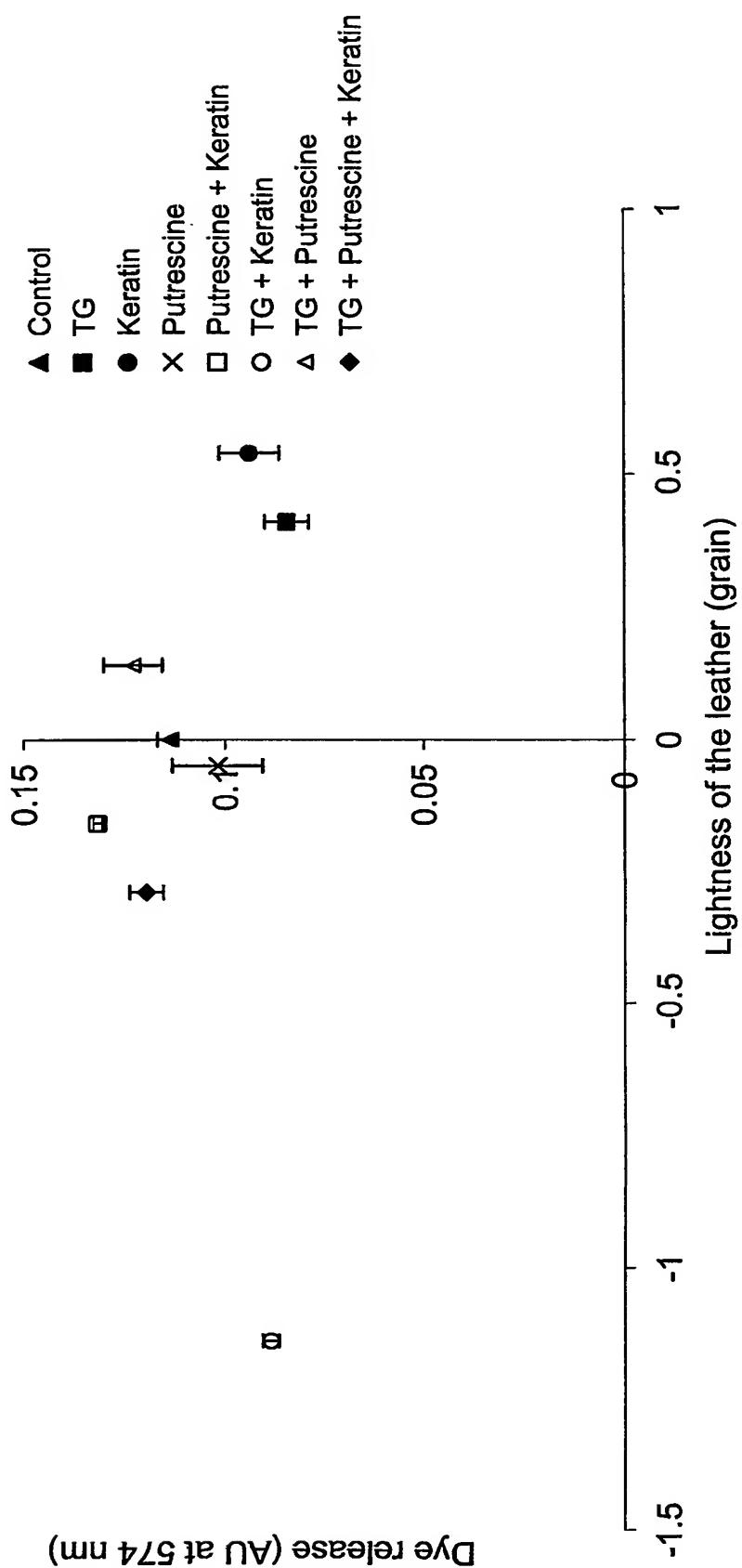


Fig. 18

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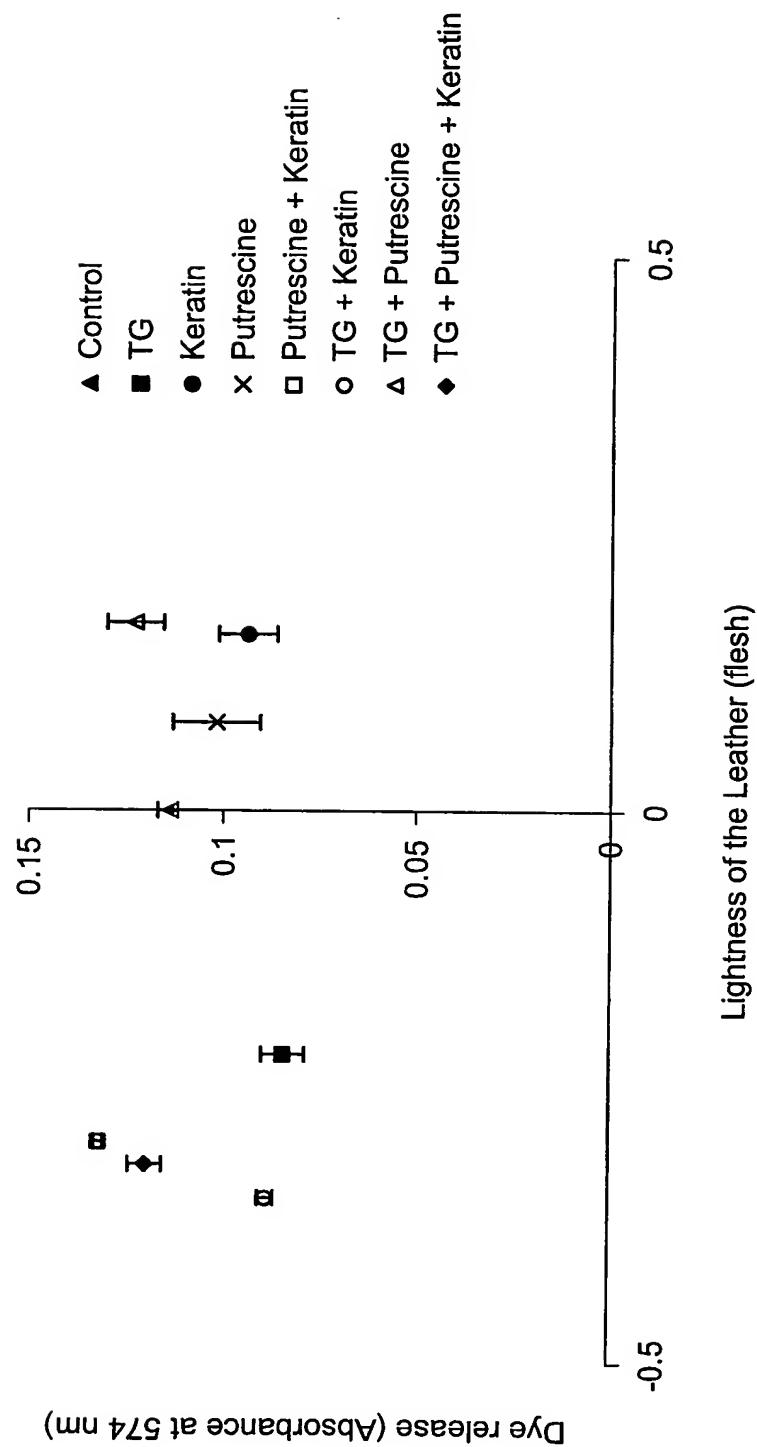
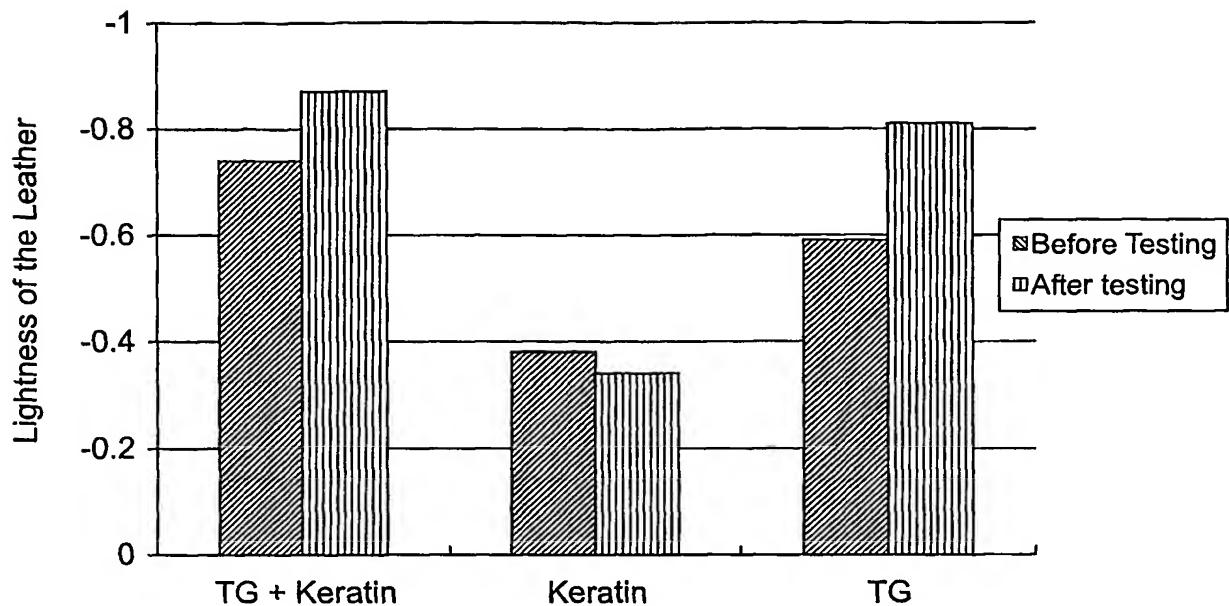
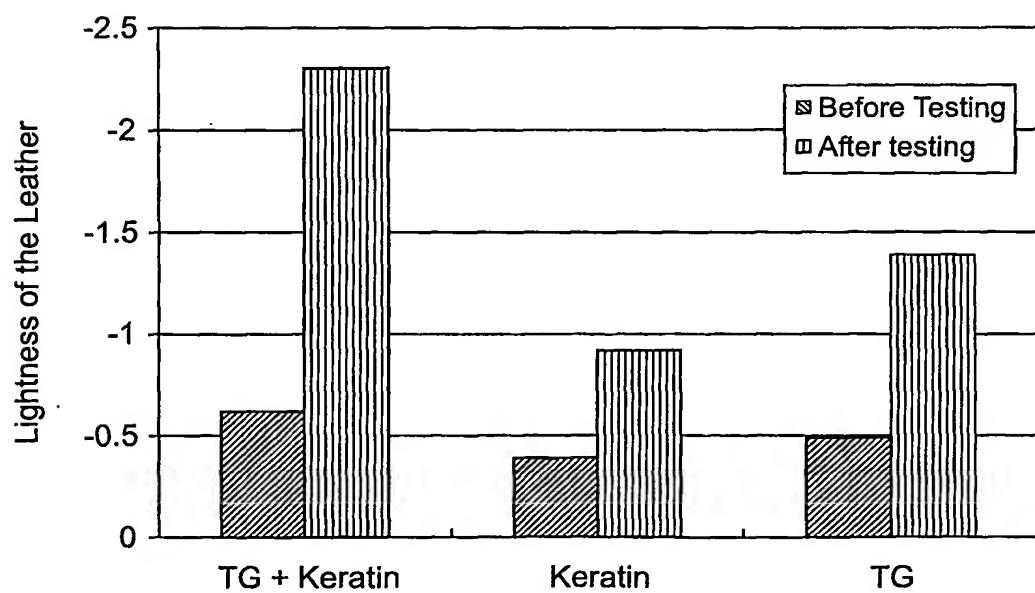
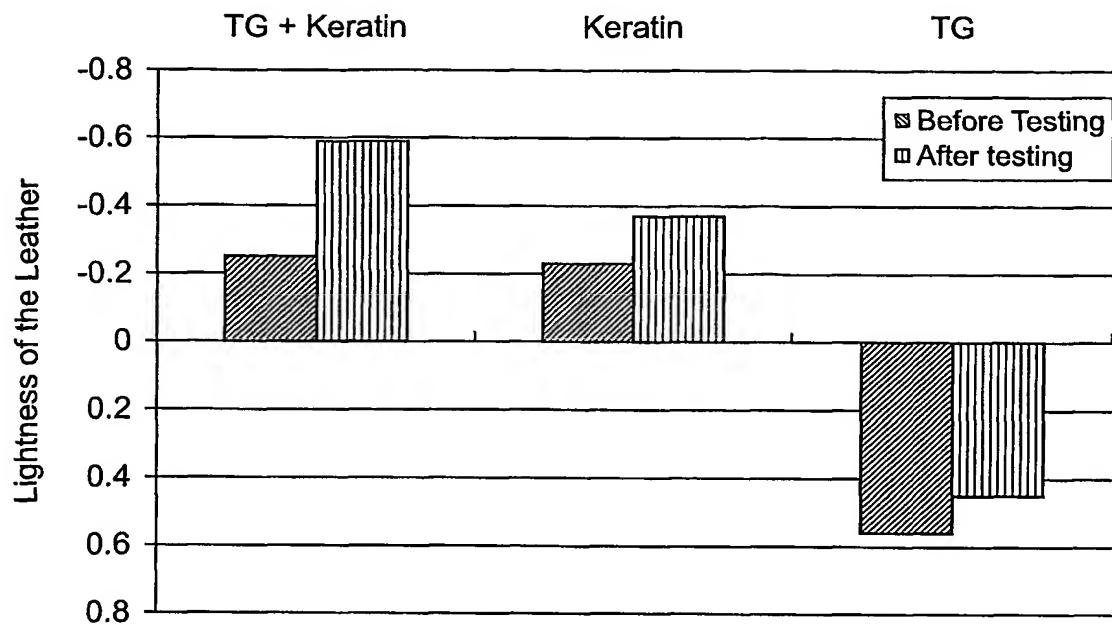
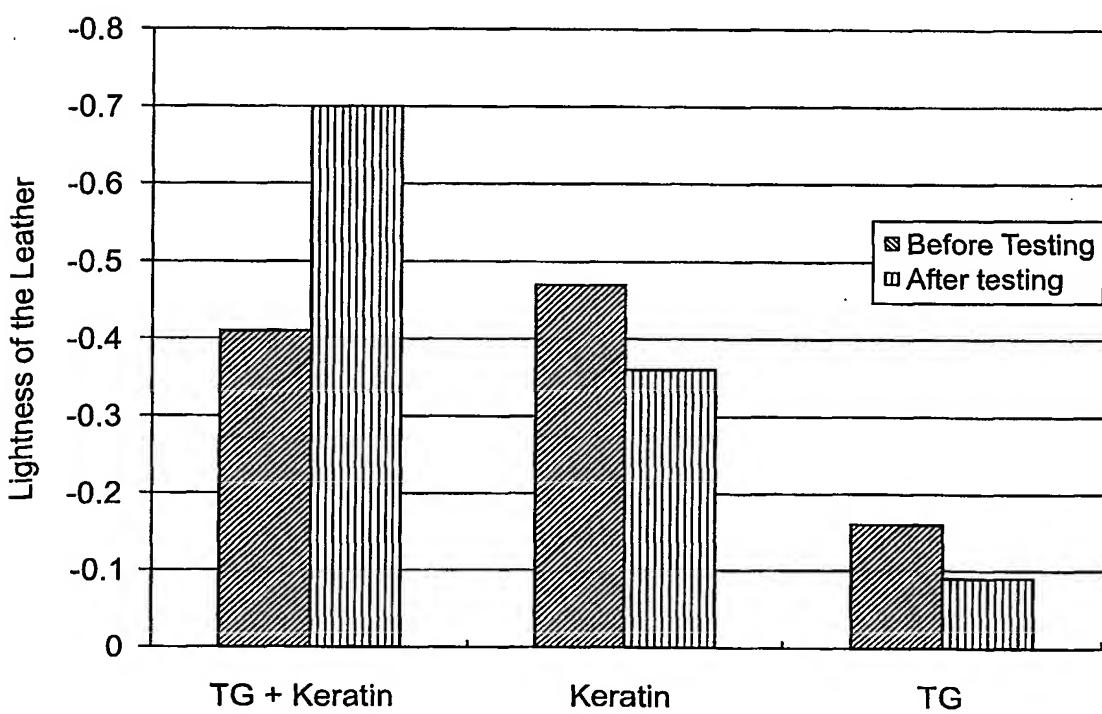


Fig. 19

20/23**Fig. 20****Fig. 21**

21/23**Fig. 22****Fig. 23**

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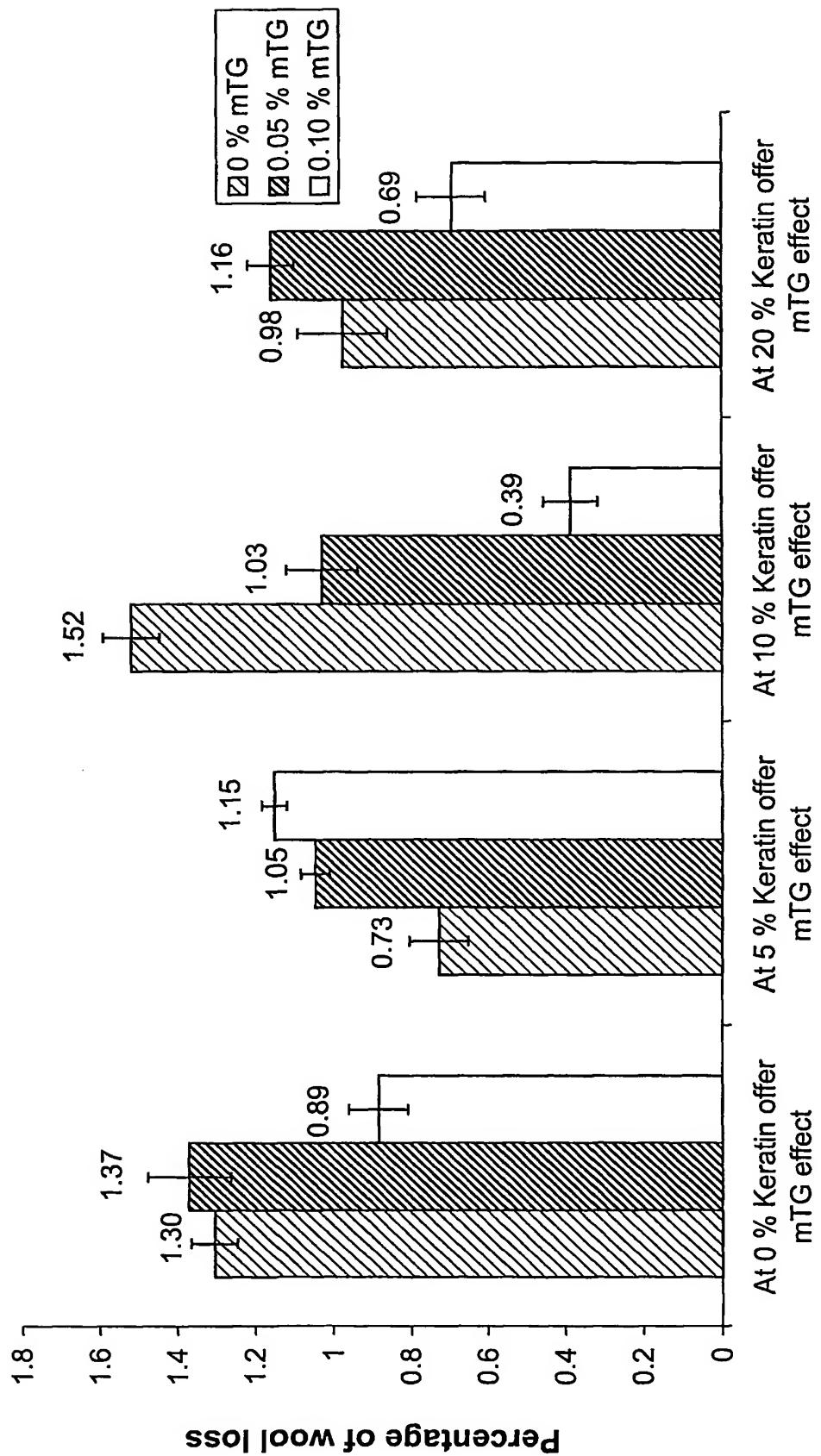


Fig. 24

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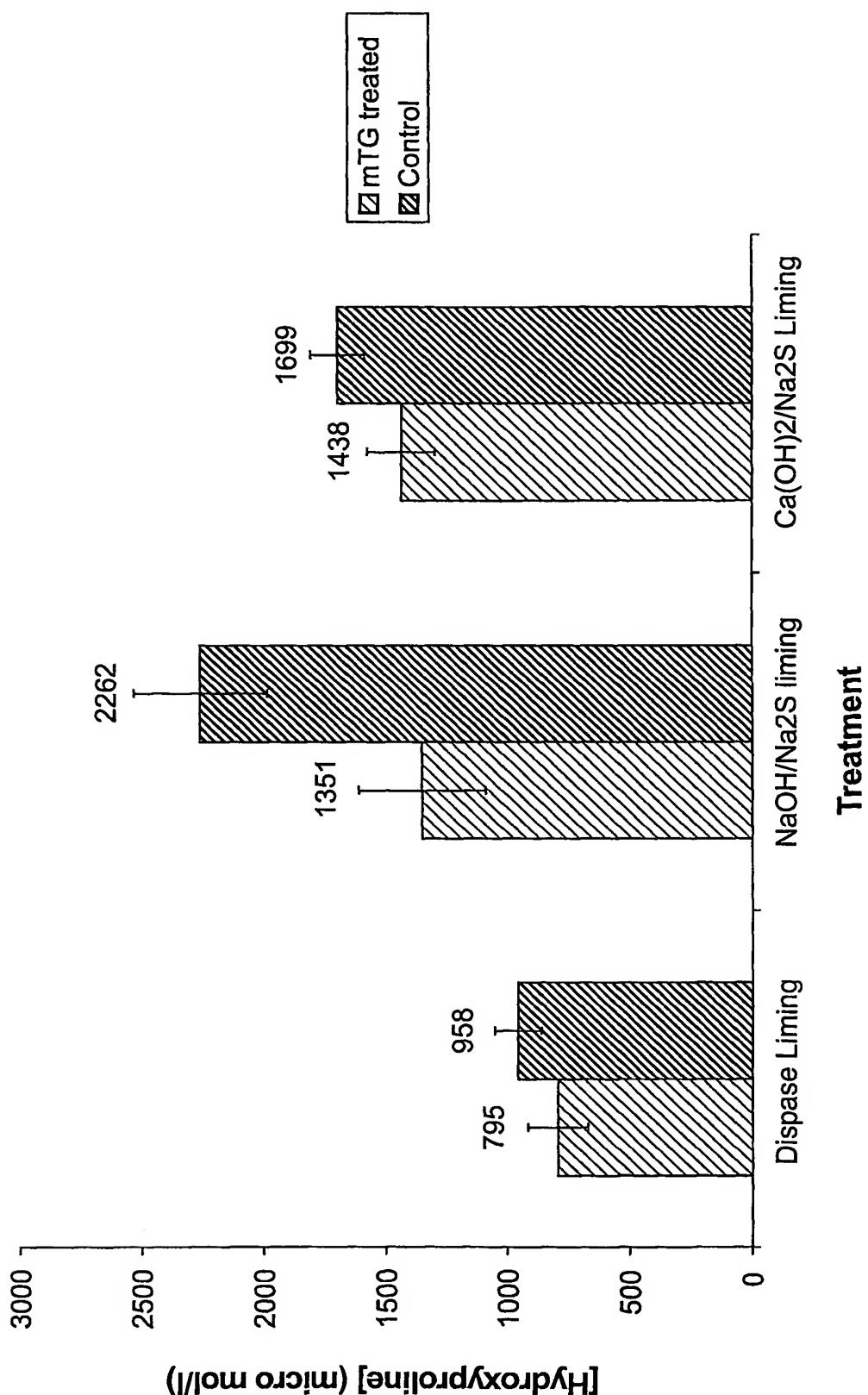


Fig. 25

INTERNATIONAL SEARCH REPORT

Application No
GB2004/003076A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C14C1/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C14C

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>DATABASE WPI</p> <p>Section Ch, Week 197629</p> <p>Derwent Publications Ltd., London, GB;</p> <p>Class D16, AN 1976-55586X</p> <p>XP002299939</p> <p>& SU 487 119 A (SHOE-LEATHER IND RE)</p> <p>4 January 1976 (1976-01-04)</p> <p>abstract</p> <p>-----</p> <p>US 2002/155524 A1 (GERMANN HEINZ-PETER ET AL) 24 October 2002 (2002-10-24)</p> <p>page 1, paragraph 11 - column 2, paragraph 19</p> <p>claims 8,9,12,13</p> <p>-----</p> <p>-/-</p>	11,16,17
X		1-3,10

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X	US 5 531 795 A (RASMUSSEN LARS ET AL) 2 July 1996 (1996-07-02) column 1, line 23 - column 2, line 61; example 1 _____	1, 3, 4, 8, 9
A	PATENT ABSTRACTS OF JAPAN vol. 1998, no. 10, 31 August 1998 (1998-08-31) & JP 10 130699 A (WAKO:KK), 19 May 1998 (1998-05-19) abstract _____	1-19

INTERNATIONAL SEARCH REPORT

Application No
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			AT	264926 T		15-05-2004
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